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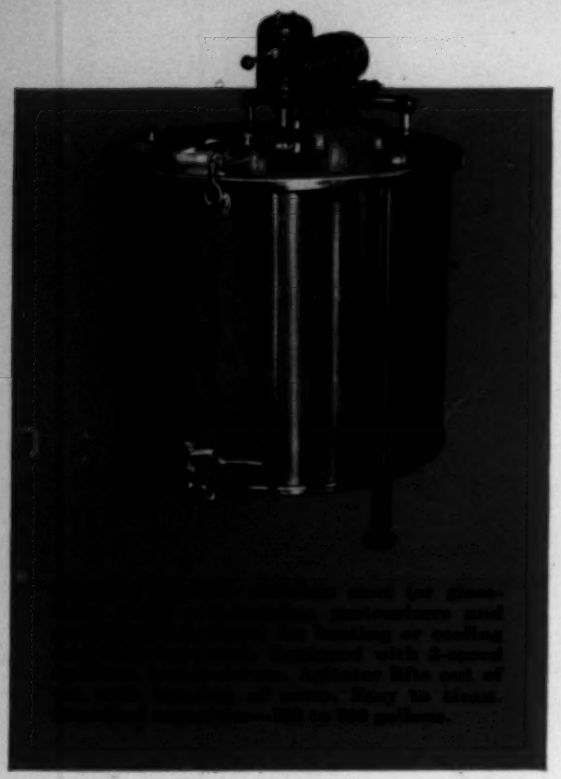

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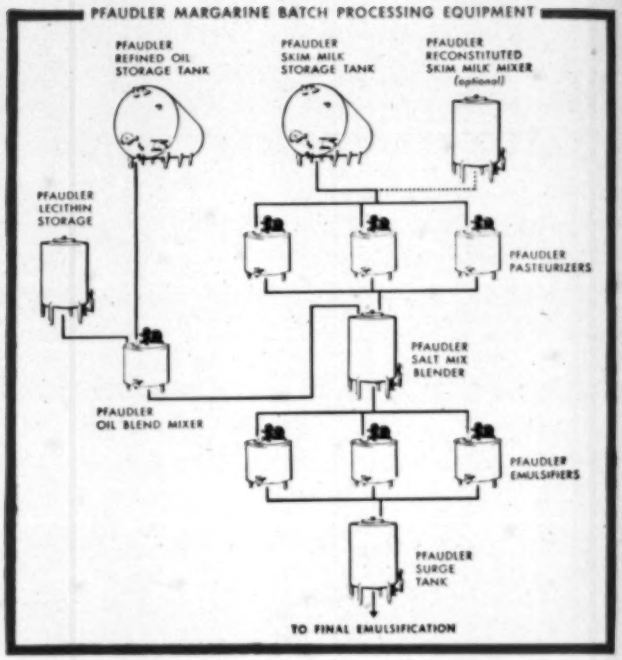

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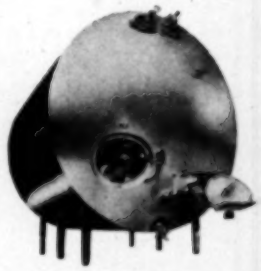

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Mushroom Mycelium Production by Submerged Propagation[a]

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A process for the production of mycelium of the commercially grown mushroom, Agaricus campestris, by submerged propagation is described. Mushroom mycelium with good mushroom flavor and also with hardly any flavor can be produced under variable conditions. The composition of the product, as compared with fresh mushroom, and its potential uses are discussed.

A preliminary paper on the growth of the mycelium of the commercially grown mushroom, *Agaricus campestris*, has been published *(2)*. The procedure now has been developed sufficiently that it seems advantageous to present a more detailed report.

The mycelium has been grown in suitable media in 100-ml. aliquots in 250-ml. Erlenmeyer flasks incubated on a shaking at 25° C., in 2-liter amounts in Fernbach flasks equipped with a stirring and aeration apparatus, and in 20-liter quantities in a fermentor described by Humfeld *(3)* for the production of Torula yeast and *Bacillus subtilis*.

The media which have been used are asparagus-butt juice, pear-waste juice, a rice bran extract, and a synthetic medium containing a suitable carbohydrate, inorganic salts, and various sources of nitrogen.

The deficiencies in the asparagus-butt juice, the pear-waste juice, and the rice bran extract have not at present been investigated sufficiently to give detailed information as to the supplements necessary for good yields.

To obtain accurate information on nutrient requirements of the mycelium, the synthetic medium was used in these investigations. Once the nutrient requirements are known, intelligent deductions can be made on the probable suitability of any other substrate.

The detailed study on the effect of varying the nutrient elements will not be presented here. At this time it will suffice to present requirements for sugar, nitrogen, phosphorus, potassium, calcium, magnesium and an arbitrary addition of four trace elements. The medium requirements are surprisingly simple and the medium is prepared as follows (in amounts per liter unless otherwise stated):

Dextrose	50 g.
Monobasic potassium phosphate, KH_2PO_4	0.87 g.
Magnesium acid phosphate, $MgHPO_4$	0.40 g.
Calcium chloride, $CaCl_2 \cdot 2H_2O$	0.37 g.
Sulfuric acid, H_2SO_4(2N)	5.7 ml.
Trace element solution[c]	20 ml.

The medium is adjusted to pH 4.5 with NaOH and sterilized at 15 pounds steam pressure for 20 minutes.

The nitrogen source was urea. This was made up in a 20 percent solution by weight, and sterilized by passage through a sterile Seitz filter. For each liter of sterilized medium, 15 ml. of urea solution was added aseptically. For a 5 percent sugar medium the concentrations of the inorganic elements in parts per million are as follows: nitrogen 1500, phosphorus 250, potassium 250, calcium 100, sulfur 100, magnesium 40, iron 4, manganese 4, zinc 2, and copper 0.5.

The medium was sterilized either directly in the flasks or fermentors or in the case, when the fermentors were refilled after the harvesting of a preceding crop of mycelium, the medium was sterilized in 4-liter bottles which were provided with an outlet tube at the bottom of the bottle.

Most of the nutrient-requirement studies were carried out in 250-ml. Erlenmeyer flasks incubated on a shaking machine. The final tests of suitability of medium for good mycelium yields under aeration-agitation conditions were made in the Fernbach fermentors or in the larger 20-liter fermentors.

The present procedure is as follows: Growing mycelium is removed from a potato dextrose agar slant with a small sterilized spatula and is introduced aseptically into a Fernbach fermentor containing 2 liters of the medium. The stirring motor and the sterile air supply are connected and the flasks are incubated with constant stirring and aeration at 25° C. until a good heavy growth has been obtained. This usually requires 5 to 6 days. The Fernbach fermentor is shown in Figure 1.

FIG. 1. Mushroom mycelium growing in Fernbach flasks fitted up as fermentors.

This culture is used as the inoculum for the larger fermentor containing 20 liters of medium. Figure 2 shows this fermentor. The medium is stirred with an agitator run at 400 r.p.m. The aeration rate is 20 liters of air per minute, or one volume of air per minute per volume of culture medium. More recently it has been discovered that a lesser number of contaminations and a somewhat increased rate of growth resulted when the aeration rate was increased to 2 or 3 volumes of air per minute.

[a] Presented before the Ninth Annual Meeting of the Institute of Food Technologists in San Francisco, California, July 13, 1949.

[b] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Report of a study made under the Research and Marketing Act of 1946.

[c] The trace element stock solution was made up as follows: $FeCl_3 \cdot 6H_2O$, 0.5 g.; $MnCl_2 \cdot 4H_2O$, 0.36 g.; $ZnCl_2$, 0.20 g.; and $CuSO_4$, 0.05 g.; water to make 1 liter.

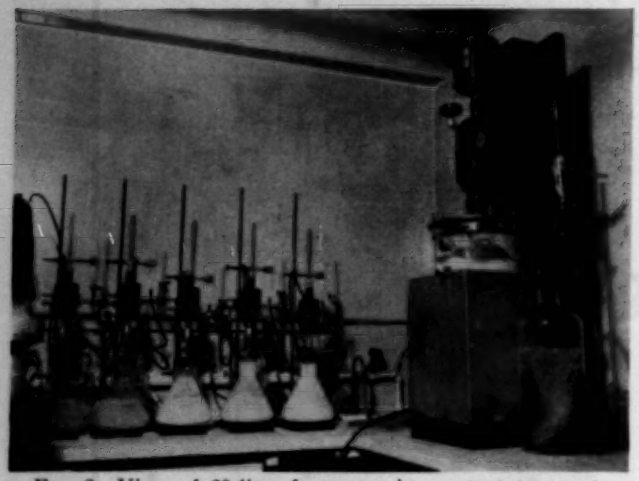

FIG. 2. View of 20-liter fermentor in constant temperature water bath and showing motor and transmission assembly.

The first harvest from this fermentor can be made in 4 to 5 days, with subsequent harvests at intervals of two to three days. It has also been discovered that to obtain a mycelium yield with good strong mushroom flavor it is necessary to continue the fermentation 1 to 2 days after all of the sugar has been consumed and the maximum amount of mycelium has developed.

Certain changes, believed to be autolytic, occur which enhance the flavor of the mycelium. The development of sufficient flavor is used to determine the time of each harvest. If the mycelium is harvested at the time of sugar disappearance and highest yield of mycelium, subsequent harvests have less and less mushroom flavor, and ultimately a practically bland product can be obtained. If it is desirable to obtain a product only for its vitamin and protein content, it may be desirable to follow this procedure.

After the harvest, the mycelium is centrifuged to remove the culture liquor, and washed with a small amount of water. It is then in a paste with the approximate consistency of a fresh yeast cake.

The yield is about 100 grams of moist mycelium (approximately 20 percent solids) per liter of a 5 percent sugar medium. Two strains of *Agaricus campestris* have been grown, one the mycelium of the white variety (strain M_5) and the other the mycelium of the brown or cream variety (strain M_{16}). The two differ in flavor, the mycelium of the brown variety possessing a somewhat nut-like flavor.

Sources of nitrogen that have proved satisfactory include alfalfa press juice, monosodium glutamate, peptone, amino acids, urea, and ammonium hydroxide. With urea as the source of nitrogen, a number of carbohydrates have given good yields. Included are *d*-glucose, *d*-galactose, *d*-mannose, *d*-fructose, maltose, *d*-xylose *l*-arabinose, dextrin, mannitol, sucrose, and soluble starch. Lactose, *l*-rhamnose and sodium carboxymethyl cellulose were not utilized.

The composition of the mycelium compares very favorably with that of the commercial mushroom (Table 1). The vitamin content is compared with that of baker's yeast in Table 2.

After harvest the mycelium can be processed in a number of ways. It can be blanched or pasteurized by heating in flowing steam in thin layers for about five minutes and then packaged and frozen as is customary for vegetables. It can be canned either as a paste or mixed with an equal weight of a one percent salt solution and processed at 15 pounds steam pressure for 20 minutes in small, 8-ounce, mushroom cans.

TABLE 1

Comparison of the Composition of the Mycelium Grown By Submerged Propagation and That of Commercially Grown Mushrooms (Agaricus Campestris)

(Calculated to a moisture-free basis)

	Commercial Mushrooms [a]	Mushroom, White Variety	Mushroom, Brown Variety
	%	%	%
Protein (N x 6.25)	37.52	35.5	45.3
Fat (ether extract)	1.81	3.3	....
Nitrogen-free extract (carbohydrate and starches)	38.19	48.8	....
Fiber	10.38	6.92	....
Ash	12.00	4.59	5.24
Ca	0.023	0.12	0.106
P	1.42	1.28	1.24
Fe	0.019	Trace	Trace

[a] Anderson and Fellers *(1)*.

TABLE 2

Comparison of the Vitamin Content of Commercially Grown Mushrooms, Baker's Yeast, and That of Mushroom Mycelium Grown By Submerged Propagation

(Micrograms per gram, calculated to a moisture-free basis)

	Commercial Mushrooms [a]	Mushroom, White Variety	Mushroom, Brown Variety	Baker's Yeast [b]
	%	%	%	%
Thiamine	11.4	8.7	8.7	24-39
Riboflavin	50	47	90	40-80
Niacin	560	190	290	280-480
Ascorbic acid	820	trace	trace	....
Carotene	none	none	none	....

[a] Anderson and Fellers *(1)*.
[b] Peterson *(4)*.

The mycelium has been spray-dried in a small experimental spray drier and drum-dried on a drier with 12-inch chromium plate rolls at 30 pounds steam pressure and a rotation of approximately 3 r.p.m. The drum-dried material seemed to have a flavor superior to that of the spray dried; however, method of drying needs further experimentation.

The processed mycelium has been used in soups, as a flavoring in sauces, and as flavoring in meat dishes and spaghetti. The time of harvest has proved to be very important for the production of the best flavor and has been determined almost entirely by cooking and tasting samples taken at some time after all of the sugar in the medium has been consumed.

With the information now available, plans include further investigation of the suitability of a number of natural substrates obtained from wastes or byproducts of agricultural origin.

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A Defrosting Indicator for Frozen Foods[a]

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A new defrosting indicator for frozen foods is described. Its action depends on the melting of a compound containing a red dye, and diffusion of the compound along a strip of filter paper as the package defrosts. At about the time of complete defrosting the red color reaches a visible position. The indicator can be adjusted to react at lower temperatures. The device is simple in design, flexible and practical in application, and inexpensive to manufacture.

Frozen foods are not sterile nor are their enzymes entirely inactivated in processing, but as long as they are maintained at a sufficiently low temperature, both microbial and enzymic activities are negligible. However, if frozen foods become defrosted, or even if they reach certain subfreezing temperatures without defrosting, deterioration in quality can occur. In fact, defrosting of some frozen foods, even when followed by immediate refreezing, may cause damage. At present there is no simple way of telling at the time of purchase whether or not a package of frozen food has been subjected to undesirable temperature.

The need, therefore, has been recognized for a device which would indicate whether or not defrosting, or exposure to undesirable temperature, has occurred. Such a device would be useful in fixing responsibility for improper storage or handling. It would also tend to speed up handling operations, since each person involved in distribution would become more conscious of the fact that he was handling perishable food.

A number of such devices have been proposed. Midgley obtained a patent (U. S. Patent No. 1,917,048) on a mark of ice or frozen brine placed on the product or package. The mark loses its identity if the temperature is allowed to rise above the "danger point." Such a mark on the outside of a package would melt before the food is defrosted and would not permit necessary handling operations during distribution.

McNaught obtained patents (U. S. Patent Nos. 2,216,127 and 2,340,337) on a similar invention. His mark consists of a comestible mixed with an edible antifreeze to give a melting point near 0° F. (—17.8° C.) and is either painted or sprayed by use of a stencil directly on the foodstuff and is then covered with Cellophane. This device reacts at too low a temperature to be considered a defrosting indicator and would not permit the handling operations necessary in distribution.

Chase invented a defrosting indicator (U. S. Patent No. 2,460,215), consisting of a hard casing running from the outside of the package to the interior; the casing contains an inelastic capsule filled with water, and a wick running to the signaling matter in the upper or outer end of the housing. In freezing, the capsule is ruptured and on defrosting, the water runs up the wick to the signaling material. The device is inserted through the wall of the package before freezing and is held in place when the food freezes around it. The Chase indicator appears to have the following disadvantages: (1) the package is punctured; (2) it lends itself only to in-package freezing, and (3) it is not simple in manufacture and application.

As far as is known, none of these inventions is used commercially. The defrosting indicator to be described here has been designed to overcome the disadvantages of those mentioned above. The device consists essentially of a small quantity of solvent containing a dye deposited on one end of a strip of filter paper which is encased in a flat, transparent sleeve (Cellophane or the like) impervious to the solvent. This assembly and a cover sheet with an opening are then attached to a package of frozen food in such a way that under normal cold storage the dye does not show through the opening. However, if the temperature rises sufficiently, the solvent melts and moves along the strip, carrying the dye to the opening or visible position, thus providing a signal that the food has been defrosted.

The device is illustrated in the drawings. In Figure 1 a package of food is shown with indicator attached.

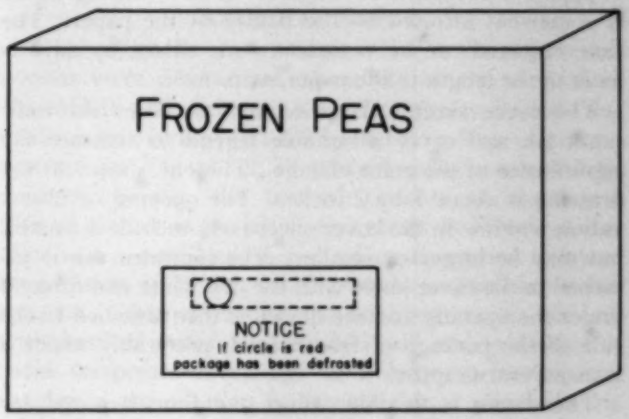

Fig. 1

Figure 2 is a cross-sectional view of the indicator on an enlarged scale. A strip of filter paper, ⅜ in. by 2 in. (No. 1 in Fig. 2) is inserted into a flat Cellophane tube (2) which extends ¼ in. beyond each end of the filter-paper strip. The strip and tube are folded midway between the ends and are tightly creased with a warm

Fig. 2

[a] Presented before the Ninth Annual I. F. T. Convention, San Francisco, Calif., July 12, 1949. A public service patent on this device is pending.

[b] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

iron. A small amount (0.05-0.07 ml.) of a solvent containing 0.25 percent of dye is inserted into the lower leg (3) of the tube, the projecting ends (4 and 5) are quickly sealed and the indicator with cover (6) is immediately placed on packages of frozen food in the cold room or stored at a low temperature until used.

Several dyes have been tested. Phenoform Red B, which gives a bright red color, is preferable with most solvents and especially with 2-phenoxyethanol or mixtures containing this compound. Flaming Red gives a dark red color and is preferred when certain solvents (hydrocarbons, etc.) are used which do not dissolve Phenoform Red B. The solvent should be a nonvolatile compound or mixture with melting point in a rather narrow range somewhat above 32° F.; viscosity and surface tension are also important factors, since these properties affect the rate of movement of the solvent through the filter paper strip. Of a number of substances investigated in a preliminary survey, 2-phenoxyethanol appeared to be most satisfactory. Further work was limited to this solvent and to mixtures containing it. Although the melting point of 2-phenoxyethanol is reported to be 14° C. (57° F.), it moves in these indicators at much lower temperatures. Its movement becomes slower as the temperature drops to 32° F.; at this temperature or slightly above, all movement ceases. From 0.05 ml. to 0.07 ml. of dye-solution is sufficient to assure development. Whatman No. 2 filter paper[c] or equivalent has proved satisfactory and the results reported below were obtained with this grade of paper. The rate of development of the warning signal is somewhat affected by the nature of the paper. The time required can be varied to some extent by adjustment of the length of the paper strip.

The cover sheet is large enough to cover the indicator tab and carry a suitable legend to indicate the significance of the color change. The one shown in the drawing is about 1 by 2 inches. The opening or observation window in the cover sheet is ¼ inch in diameter, but may be larger or smaller. The indicator tab is attached to the cover sheet with the dry white end directly under the opening and the device is then attached to the side of the package of frozen food, preferably under a transparent wrapper.

The device is flexible. Since the time required for the indicator to develop at a given temperature depends on the nature and length of the paper strip and the physical properties of the dye solvent employed, it is obvious that the time required can be varied as described. In a number of experiments it was found that a 12-oz. package of frozen peas would completely defrost and the internal temperature begin to rise above 32° F. in 6 to 7 hours in still air at a room temperature of 77° F. (25° C.). It was therefore the objective of several experiments to find a dye solvent that would require about 6 hours to develop at 77° F. and longer times at lower temperatures down to 32° F., below which no development would take place.

Table 1 shows how indicators develop when 2-phenoxyethanol is the solvent and how the rate of development is affected by mixing other compounds with it. 2-phenoxyethanol works fairly well at temperatures from 34° F. up, with no development below that temperature. Glycerol (100 percent) is too slow at 77° F. and 60° F. but might be useful on large packages or cases which take longer to defrost. Didecyl ether and hexadecane would not develop at 40° or 34° F. Undecanol develops in less than 20 hours at 0° F., although its melting point is reported to be 12° C. (53.6° F.). None of the solvents or mixtures listed in Table 1 moved in the indicators at 0° or 15° F. in 75 days except 20 percent undecanol in 2-phenoxyethanol, which developed in 17 days at the latter temperature.

TABLE 1

Hours Required for Development of Visible Color at Various Temperatures

Dye Solvent (or Activator)	77° F. Hours	60° F. Hours	40° F. Hours	34° F. Hours	15° F. Hours
2-phenoxyethanol	6.5	10.0	48.0	109.0	X
5% undecanol in 2-phenoxyethanol	7.3	9.6	37.1	84.0	X
10% undecanol in 2-phenoxyethanol	5.2	10.5	24.5	101.0	X
15% undecanol in 2-phenoxyethanol	4.6		22.0	51.0	
20% undecanol in 2-phenoxyethanol	3.8	8.5	15.8	50.0	17 (days)
4% lauric acid in 2-phenoxyethanol	6.0	10.7	70.7	X	X
10% castor oil in 2-phenoxyethanol	6.0	12.0	41.7	X	X
33% castor oil in 2-phenoxyethanol	6.9	12.2	46.0	121.0	X
5% glycerol in 2-phenoxyethanol	7.1	12.7	63.6	109.0	X
10% glycerol in 2-phenoxyethanol	7.6	11.7	34.6	X	X
10% butyl stearate in 2-phenoxyethanol	7.8	12.3	85.6	109.0	X

X No movement of dye solution in the paper strips in 75 days.

The defrosting indicator has been designed primarily for individual packages. Defrosting within cases could be ascertained by having a small Cellophane window located in the case directly over an indicator of one of the individual packages.

No attempt has been made to suggest various ways in which the indicator may prove useful in transportation, storage, distribution, and retail sale of frozen foods. It seems probable, however, that it can be adapted to a range of uses and purposes, with advantage to various commercial handlers of frozen foods, from packer to consumer. Indicators that apply the principles and mechanism described can be used not only to reveal defrosting but also to show whether or not temperatures somewhat lower than freezing have been reached for specific periods of time.

[c] Mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

Enzymatic Oxidation of Phenolic Compounds in Frozen Peaches[a]

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Methods are presented for the quantitative estimation of rate and extent of enzymatic browning in frozen peaches. The degree of browning in several peach varieties was found to be directly related to the amounts of oxidizable tannins present.

The problem of browning in frozen peaches is one of long standing and has been the subject of numerous articles and reports. The process is considered to be enzymatic in nature and due to oxidation of so-called catechol tannins by oxidizing enzymes in the presence of oxygen.

As early as 1920, Onslow *(8)* showed that peaches contain both oxidizing enzymes and certain phenolic compounds, the latter being converted to brown-black substances of unknown constitution during the oxidation. By means of qualitative tests, Kertesz *(7)* showed in 1933 that the oxidizing enzymes of Sunbeam peaches were similar to those of other peach varieties. He concluded that the low concentration of catechol tannins in this variety was responsible for the failure of the sliced fruit to darken. In 1939, Saburov and Kaperina *(11)* determined the tannin content of several Russian Peach varieties by titration with potassium permanganate. They correlated the resulting tannin values with visual color observations on the cut slices, and on the basis of these results, classified the known varieties into four groups, according to relative browning tendencies. Blake and Davidson *(3)* classified a number of varieties on the basis of the color reaction between 4 ml. of peach juice and 3 drops of 6 percent $FeCl_3$ solution. They estimated the tannin by the color and thickness of the discolored layer formed by adding the $FeCl_3$ to the peach juice. On this basis they found that varieties high in acidity as well as in tannin were objectionably astringent or unpalatable.

The literature is replete with methods and procedures concerned with the inhibition or elimination of the undesirable color changes caused by oxidation of phenolic compounds during processing, freezing, and thawing of cut tree fruits *(1, 2, 4, 5, 6, 12, 13, 14, 15, 16, 17, 18)*. However, there is a lack of quantitative data such as are required to demonstrate the relationship between browning and the components which are responsible for the browning reaction.

This paper is primarily concerned with the quantitative evaluation of rate, as defined herein, and extent of enzymatic browning in several varieties of peaches. The quantitative relationships shown may be used to classify peach varieties as to their total browning susceptibilities.

[a] Presented before the Ninth Annual I. F. T. Convention, San Francisco, Calif., July 14, 1949.

[b] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

Procedure and Preparation of Materials

The samples were obtained from various parts of California and Washington, and consisted of more than 50 varieties and hybrids. The hybrids and varieties originated chiefly at the University of California Experimental Farm at Winters, Calif. In most cases each sample was harvested from a single tree. Immediately after harvesting and transporting to the Western Regional Research Laboratory by truck, the whole unpeeled fruit was frozen in covered 30-pound cans at —10° F. The samples from the Irrigation Branch Experiment Station at Prosser, Washington, were frozen within a few hours after harvesting and subsequently shipped to the Western Regional Research Laboratory in an insulated container refrigerated by dry ice.

While still frozen, at least 15 peaches from each variety were slip-peeled by quick immersion in cold water, pitted, quartered, and ground at —10° F. (—23.3° C.) to pass through a 4-mm. screen. The ground, frozen material was thoroughly mixed, packed in half-gallon friction-top cans, and stored at —10° F. All determinations were made on these uniformly mixed samples to avoid errors due to variations in individual peaches.

Analytical Methods

Total Phenolic Compounds: Total phenolic compounds were determined essentially by the method of Rosenblatt and Peluso *(10)*. The determination was made on an extract prepared by blending 50 g. of ground material in 300 ml. of 70 percent aqueous alcohol by volume. Briefly, the method consists of treating a filtered aliquot of the alcoholic extract in 75 ml. of distilled water with 2 ml. of Folin Dennis reagent followed by addition of 20 ml. of 10 percent sodium carbonate solution. After adjusting the final volume to 100 ml. with distilled water, the sample is allowed to remain at room temperature for 1½ to 2 hours before optical density of the solution is measured in a photo-electric colorimeter equipped with a 660 mμ filter. The method was standardized against a sample of pure tannic acid and all results are expressed and reported on the basis of anhydrous tannic acid.

Water, 95 percent alcohol, 70 percent alcohol, and aqueous 0.3 percent oxalic acid solutions were tried as solvents for the tannin extraction. The latter three were chosen in an attempt to prevent the browning which was clearly visible during the blending with water. Since a 70 volume percent alcohol-water mixture gave the highest values for total phenolic compounds from peach tissue (Table 1), all actual tannin extractions from peaches were carried out with this solvent. The fruit or fruit-water mixture was extracted for 3 minutes in a blender with 6 volumes of extractant for each part of peach or peach-water mixture. These proportions gave a final alcohol concentration of approxi-

TABLE 1

Total Tannin Extracted from Peach Tissue by Various Solvents

Variety	Tannin as tannic acid (mg./100 g.)			
	Distilled water	0.3% aqueous oxalic acid	95% alcohol	70% alcohol
Late Hale	62	84	90	108
Lovell	....	79	88	99
Elberta	....	60	....	75
Goldeneast	....	53	....	67
W31-49C	....	56	....	69
Sunbeam	....	23	....	28

mately 60 percent, which was sufficient to eliminate turbidity in the extracts as well as oxidation of phenolic compounds during blending.

Enzyme Activity: Phenolase enzyme activity was determined essentially by the methods of Ponting and Joslyn *(9)*.

Estimation of Rate of Tannin Oxidation: 150 g. of ground frozen peach tissue and 150 g. of distilled water are rapidly homogenized in a blender in less than one minute. Initial total phenolic compounds are immediately determined (before any browning takes place) on 50 g. of the mixture as described in the previous section. The blender is allowed to mix the remaining homogenate at high speed for 10 minutes, which is sufficient to bring the temperature to 77° F. (25° C.). Thereafter the blending speed is reduced by means of a variable transformer so as to keep the temperature of the mixture in the range of 77° F. (24 to 26° C.) and also allow adequate aeration of the tissue. At 15-minutes intervals, additional 50-g. portions are removed and analyzed for total phenolic compounds.

The progress of the oxidation can be followed by subtracting the residual tannin value at each time interval from the original tannin value of the non-browned mixture. From these data the rate of oxidation, expressed as milligrams of tannic acid oxidized per 100 grams of material per 15-minute interval, can be calculated. The brown enzymatically oxidized compounds are either unable to reduce the Folin Dennis reagent to its characteristic blue color or else they contain only a small fraction of the free phenolic groups originally present in the unoxidized peach tissue.

In order to show that the tannin oxidation in frozen peaches is due to enzymatic action rather than to autoxidation, heated and unheated samples of the same peach tissue were allowed to oxidize for one hour under controlled conditions. The sample which was heated to inactivate the enzyme system did not show any loss of tannin during the reaction period, while the unheated sample lost 75 percent of its original tannic acid equivalence (Table 2). Furthermore, this decrease in total phenolic compounds in the unheated sample correlated rather well with increasing darkening as judged visually, whereas the heated sample showed no browning tendency whatsoever.

TABLE 2

Effect of Enzymes on Amounts of Tannin Oxidized in Peach Tissue

Reaction time (min.)	Enzymatically active (no treatment)		Enzymatically inactive (boiled ten minutes)	
	Total tannin [a]	Tannin oxidized [a]	Total tannin [a]	Tannin oxidized [a]
0	144	0	144	0
30	51	93	143	....
60	36	108	145	....

[a] As tannic acid, mg./100 g.

Relationship Between Oxidation of Phenolic Compounds and Color Changes in Peach Tissue

Since visual observations of color changes occurring in frozen peaches are not easily reproducible and are always subject to large human errors, it was necessary to devise an objective method for the determination of the relationship between tannin oxidation and color changes in mixture of peach tissue and water.

Water extracts were prepared by rapidly homogenizing frozen peach tissue with an equal weight of distilled water and squeezing the mixture through a milk filter disk. (Numerous experiments have shown that this procedure extracts 80 to 85 percent of the tannin extractable by the 70:30 alcohol-water mixture described earlier.) The homogenization and filtration were carried out before any browning took place. Two ml. of the filtrate was pipetted into 20 ml. of distilled water in a colorimeter tube (2.2 cm. in diameter), and after thorough shaking, the optical density of the solution was measured in a photoelectric colorimeter equipped with a 420-mμ filter.

An aliquot for tannin analysis was removed at the same time, and total phenols were determined in the usual manner. The filtrate was rapidly warmed to 77° F. (24° to 26° C.) by agitation in a water bath, and at definite time intervals additional samples of the filtrate were tested for both tannin and optical density. It should be pointed out that extracts filtered through milk filter disks are by no means optically clear solutions. The turbidity factor was minimized by use of the same volume of the same well-agitated filtrate for each of the measurements.

When the amount of oxidized tannin at definite reaction times is plotted against the corresponding change in optical density of the browning filtrate, a straight-line relationship is obtained (Figure 1). Mathematical treatment of the data indicates that the browning proceeds according to a first-order reaction, particularly after the initial fifteen minutes of aeration (Figure 2).

Further data on the relationship between tannin oxidation and color change were obtained by determining

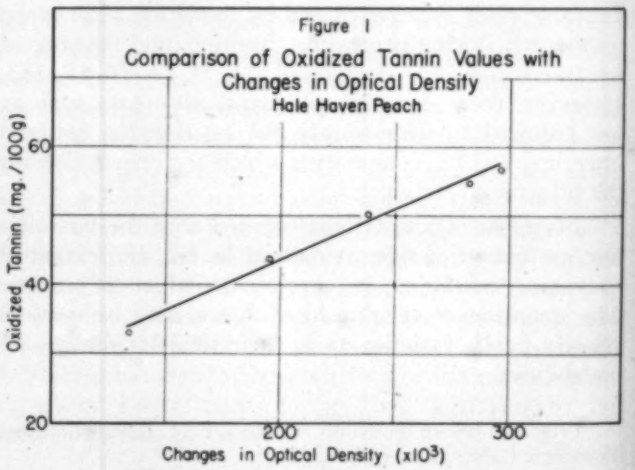

FIG. 1. Comparison of Oxidized Tannin Values with Changes in Optical Density. Hale Haven Peach.

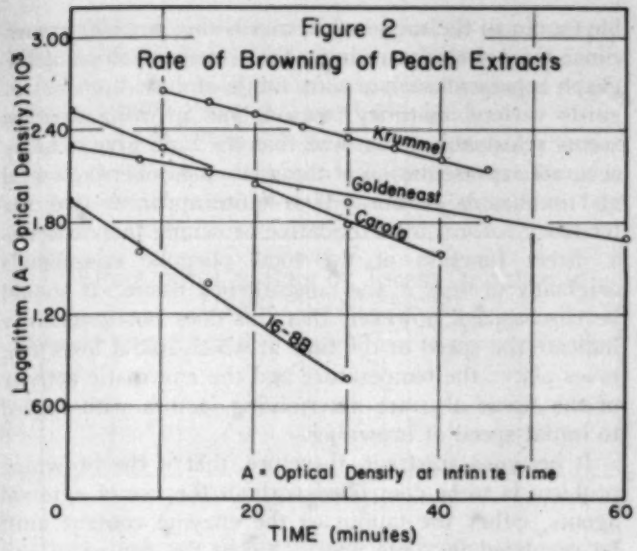

FIG. 2. Rate of Browning of Peach Extracts.

the rate and extent of tannin oxidation and optical density change in several varieties of frozen peaches. Each method of following the rate of browning was employed on separate portions of the frozen material.

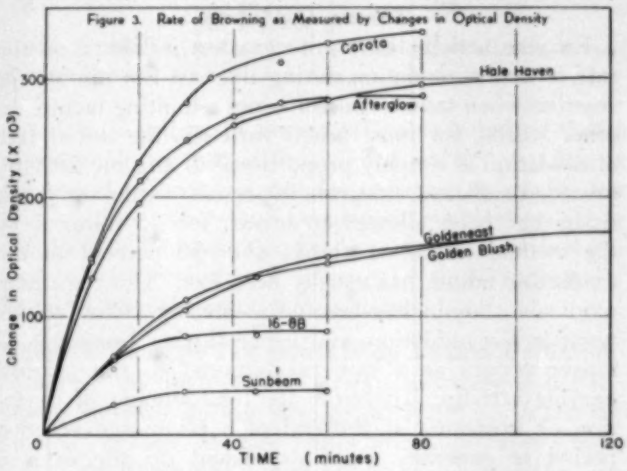

FIG. 3. Rate of Browning as Measured by Changes in Optical Density.

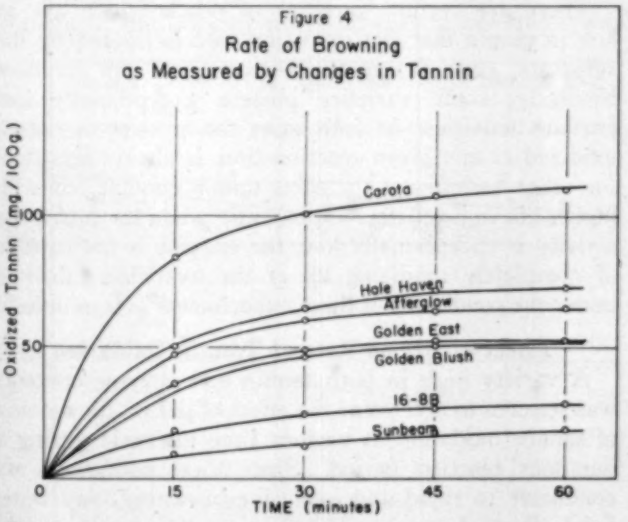

FIG. 4. Rate of Browning as Measured by Changes in Tannin.

Examination of the data in Figures 3 and 4 shows that the shape of the curves representing the rate of browning are similar for both methods of evaluation. Figure 5 shows a fairly close linear relationship between total color change, as measured by total change in optical density, and total phenolic compounds oxidized, ex-

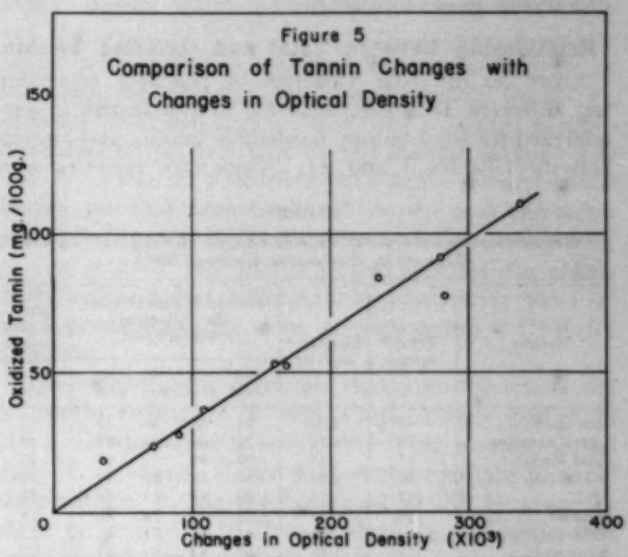

FIG. 5. Comparison of Tannin Changes with Changes in Optical Density.

TABLE 3

Relationship Between Total Tannin, Enzyme Activity, and Tannin Oxidized in California Peaches (1947)

Variety	Total[a] tannin	Enzyme[b] activity	Tannin oxidized[a] after the following reaction times:				Percentage of total tannin oxidized in 60 min.
			15 min.	30 min.	45 min.	60 min.	
16-14B	32	.19	11	13	15	18	56
W21-22C	36	.32	14	....	....	18	50
Sunbeam	35	.42	9	12	17	19	54
20-9B	40	.29	10	16	18	19	47
Rio Oso	40	.71	13	18	21	22	55
Dripstone	46	.78	16	20	22	22	48
17-9D	39	.26	15	20	22	23	59
17-9B	43	.48	19	24	26	27	63
W3-16	42	.74	21	24	27	27	64
16-8B	41	.38	18	24	26	27	66
Triogem	55	.10	17	27	31	32	58
Alamar	53	.97	19	30	34	35	66
17-5D	55	.35	21	30	35	36	65
15-7C	62	.45	31	39	....	42	68
Elberta	67	1.23	41	47	47	48	72
Newday	84	.10	18	40	43	48	57
Sun Gold	70	.81	31	42	44	49	70
Summercrest	70	.48	34	46	....	50	71
Primrose	74	.81	35	44	48	51	69
Amador	74	.58	39	47	49	51	69
W31-49C	72	.61	....	48	....	52	72
Golden Blush	75	.77	36	46	51	52	70
Golden East	75	.29	36	48	50	52	70
July Elberta	78	.18	41	51	54	55	71
W29-35B	82	.48	36	50	54	57	70
14-5	87	.26	33	50	57	60	69
Afterglow	85	.68	50	60	63	64	75
Sunhigh	85	.32	48	55	61	64	75
J. H. Hale	86	.65	51	62	64	65	75
15-5C	95	.77	37	59	66	68	72
Vee-Freeze	94	.48	43	66	69	70	75
Hale Haven	99	.68	48	64	71	71	72
W24-29	97	.93	49	66	72	72	74
Lovell	100	.64	50	65	70	73	73
W29-41C	100	.61	43	64	70	73	73
Late Hale	105	.32	27	62	70	74	72
Roberta	102	.39	56	71	75	77	75
White Hale	101	.71	59	72	76	78	77
Fay Elberta	110	1.23	75	....	86	85	77
Carota	141	.81	84	100	107	109	77

[a] As tannic acid, mg./100 g.
[b] As mg. of ascorbic acid oxidized per g. of fruit per min. at 25° C.

pressed as milligrams of tannic acid per hundred grams of material. Hence it appears that the loss in phenolic compounds as measured herein is a fairly good estimate of the degree of enzymatic browning in frozen peaches. Furthermore, this relationship need not be limited to frozen peaches, since it is equally true for fresh peaches of varying stages of maturity.

Relationship Between Total and Oxidized Tannin

Over 50 different varieties of peaches, representing different localities, seasons, and maturities, were analyzed for total tannin, oxidizable tannin, and enzyme activity (Tables 3 and 4). Numerous varieties were

TABLE 4

Relationship Between Total Tannin, Enzyme Activity and Tannin Oxidized in Washington Peaches (1947)

Variety	Total [a] tannin	Enzyme [b] activity	Tannin oxidized [a] after the following reaction times:				Percentage of total tannin oxidized in 60 min.
			15 min.	30 min.	45 min.	60 min.	
Sun Gold	49	1.50	24	30	30	32	65
Late Champion	56	1.52	31	34	36	38	68
Gold Medal	65	2.40	29	36	37	46	71
J. H. Hale	89	2.55	54	64	66	69	77
37-37	107	.70	50	68	72	76	71
Amador	105	.96	55	73	78	80	76
W31-49C	112	.97	61	66	71	81	72
Krummel	118	1.61	67	83	89	91	77
Elberta	124	.998	76	88	93	98	79
Halberta	124	2.56	82	93	98	100	80
Fay Elberta	148	1.63	89	106	112	115	78

[a] As tannic acid, mg./100 g.
[b] As mg. of ascorbic acid oxidized per g. of fruit per min. at 25° C.

allowed to brown for 24 hours by the method described herein, and in practically all cases the amount of tannin oxidized in 24 hours was not appreciably greater than the amount oxidized in one hour. On most varieties, therefore, the oxidizable tannin was determined by subtracting the tannin value after one hour of reaction from the original total tannin content of the nonbrowned tissue. For a few samples with exceptionally high tannin content and low enzyme activity, the oxidizable tannin values were determined by increasing the reaction time until no further oxidation occurred. The analytical data of Figure 6 indicate the existence of a straight-line relationship between total and oxidizable tannin in the range of 32 to 194 mg. per 100 grams. Since the points determining the shape and slope of the graph represent such a wide range of conditions as regards variety, maturity, season, and growing area, it seems reasonable to assume that the data give a fairly accurate representation of the relationship between total and oxidizable tannins. It is quite apparent that the tendency toward total oxidative browning in peaches is a direct function of the total phenolic compounds originally present in the nondarkened tissue. It should be emphasized, however, that this does not necessarily indicate the speed or the time at which initial browning takes place; the temperature and the enzymatic activity of the tissue also are determining factors with regard to initial speed of browning.

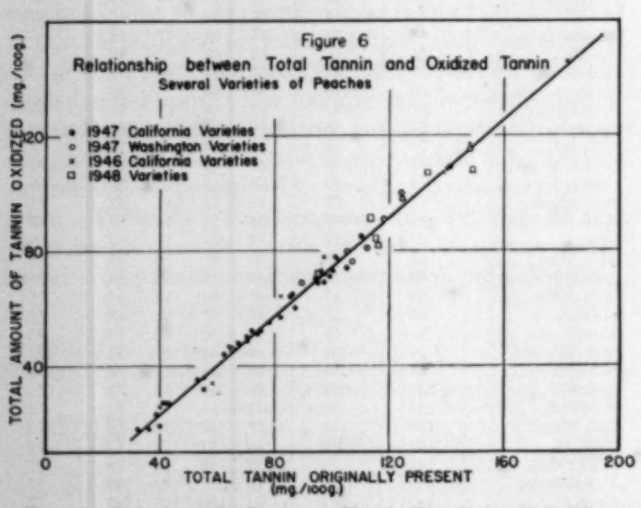

FIG. 6. Relationship Between Total Tannin and Oxidized Tannin of Several Varieties of Peaches.

It becomes apparent, therefore, that if the browning problem is to be controlled without the use of external agents, either the tannin or the enzyme content must be regulated in some way. During the course of this work it was found that varieties whose oxidizable tannin values were 18 to 19 mg. per 100 g. exhibited very little, if any, darkening on prolonged exposure to air. Thus it is possible to avoid browning by selecting varieties which are sufficiently low in oxidizable tannin.

Effect of Enzyme and Tannin Contents on Rate of Tannin Oxidation

Enzyme activity exerts its greatest influence on the rate of tannin oxidation during the first few minutes of reaction when tannin content is not a limiting factor. In other words, for most peach varieties, the initial rate of oxidation is roughly proportional to enzyme activity, provided sufficient substrate is present. After the peach tissue has been allowed to brown for 15 minutes by the method described above, over 50 percent of the oxidizable tannin has usually been lost. The remaining oxidizable tannin has become a limiting factor at this point in the oxidation, and hence further browning no longer occurs at a rate proportional to the original enzyme activity. Therefore the total amount of oxidation or browning at the end of a 60-minute reaction period is generally more dependent on the original amount of tannin present in the peach than on its enzyme activity (Tables 3 and 4).

There are certain varieties of peach which are so low in tannin that the oxidation rate is limited by the substrate, even during the beginning of the reaction. Similarly, some varieties possess exceptionally low enzyme activities; in such cases the amount of tannin oxidized at any given reaction time is always less than for other varieties of the same tannin content but with higher enzyme activity. Apparently when the phenolase activity is exceptionally low, the enzyme is not capable of completely oxidizing all of the available substrate under the conditions of these experiments (60 minutes).

Effect of pH on Rate of Tannin Oxidation

A variety high in both tannin and enzyme contents was selected to determine the effect of pH on the amount of tannin oxidation at various time intervals during a one-hour reaction period. Since these conditions are conducive to rapid and extensive browning, any beneficial effect of certain pH values on this variety might

be expected on other varieties of equal or less susceptibility to darkening. The rate of oxidation was determined on three lots of the same material adjusted to pH values of 3.8, 3.2, and 2.6 with citric acid.

The curves in Figure 7 show a very marked effect of pH on the amount of tannin oxidized, especially so at the lowest pH value. Activity measurements on the

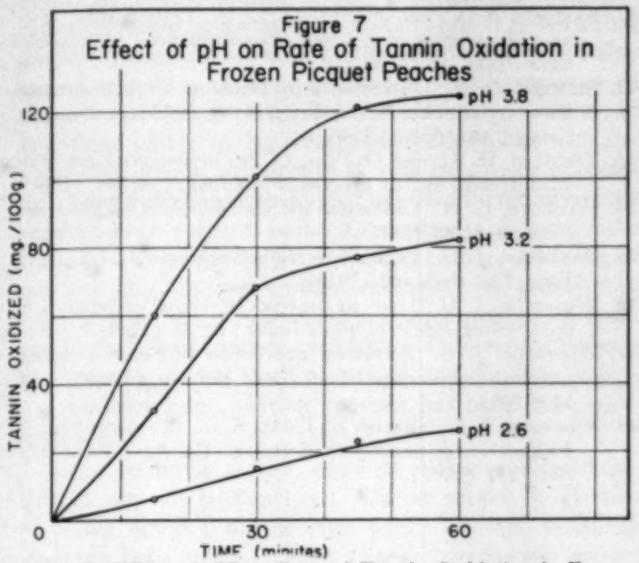

FIG. 7. Effect of pH on Rate of Tannin Oxidation in Frozen Picquet Peaches.

sample adjusted to the lowest pH value indicated that a considerable amount of the original activity was left at the end of an hour. Hence the reduction in rate is due to inhibition rather than permanent inactivation. It is well to note, however, that the lower pH values used are well below the minimum values (3.4 to 3.7) naturally found in even the most acid varieties.

Effect of Storage Temperature on Tannin Oxidation

During the 1946 season, 20 varieties were tested for tannin stability during storage at —10° F. After six months' storage at this temperature, no appreciable tannin oxidation was found in any of the varieties. In another experiment, a variety with high tannin content was stored at —10°, 0°, 10° and 20° F. The tannin content of the samples stored at each temperature was

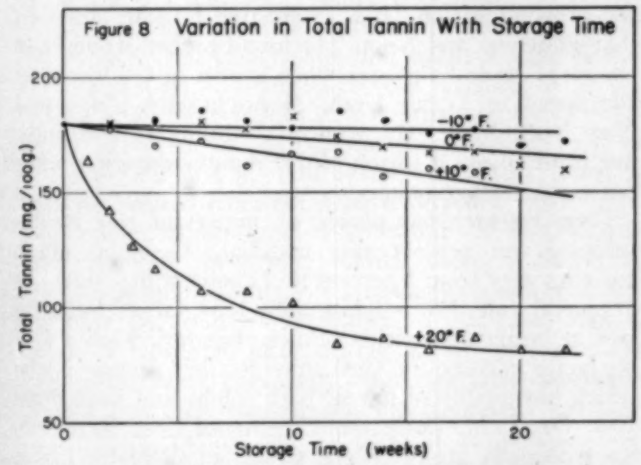

FIG. 8. Variation in Total Tannin with Storage Time.

determined at bi-weekly intervals over a period of twenty-two weeks.

The curves in Figure 8 indicate no significant change in the samples stored at —10° F. The samples stored at 0° F. appear to be undergoing oxidation at a very slow rate after an initial lag period of eight to ten weeks, during which no significant change in tannin was observed. At 10° F. the rate of oxidation is slightly higher than at 0° F., and there appears to be little or no initial lag period. The samples stored at 20° F., however, indicated a very rapid loss in tannin, particularly during the first part of the storage period. The rate of oxidation proceeded in a fairly linear fashion during the first four weeks of storage and thereafter gradually began to level off. After as little as two weeks' storage at 20° F., the frozen ground material was visibly discolored and had undergone approximately twice as much oxidation as the same samples stored at 0° F. for 22 weeks.

These relationships indicate that from the standpoint of tannin oxidation, a very short storage period at 20° F. is equivalent to a relatively long storage period at 0° F. or lower. Hence it is quite important to avoid temperatures in the neighborhood of 20° F. even for short periods of time. It is understood, however, that these experiments were carried out without the benefit of added antioxidants, which undoubtedly would serve to minimize the effects of the higher temperatures.

Summary

Methods are presented for the quantitative estimation of the rate and extent of enzymatic browning in frozen peaches.

A fairly good correlation has been established between the oxidation of phenolic compounds and changes in the optical density of aqueous extracts of peach tissue.

Only a fraction of the total phenolic substances in peaches is of the oxidizable type, but a definite correlation exists between this fraction and the total phenolic compounds present.

The susceptibility to total enzymatic browning in several peach varieties was found to be directly related to the amounts of oxidizable tannins present. Initial browning tendency, however, was also governed by the original activity of the enzymes.

Varieties whose oxidizable tannin value was in the range of 18 to 19 mg./100 g. exhibited little or no browning on prolonged exposure to air regardless of the enzyme activity.

Changes in pH markedly affect the rate of browning; the lower pH values corespond to slower browning rates.

The oxidation of the phenolic substances in peaches is markedly influenced by the storage temperature. Little or no oxidation occurs at —10° or 0° F., but significant amounts of tannin are oxidized after as little as one week of storage at 20° F.

Acknowledgments

The authors are indebted to R. R. Legault for counsel and encouragement, and to other staff members of this Laboratory who have given valuable suggestions and

assistance in preparation of the manuscript, particularly William F. Talburt and Charles G. Seegmiller. The generous cooperation of the Division of Pomology of the University of California Experiment Station at Davis in supplying most of the varieties and hybrids used in these experiments is gratefully acknowledged.

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Aspects of Modern Onion and Garlic Dehydration[a]

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(REVIEW PAPER)

The importance of proper raw material and the scarcity of suitable types are discussed. Since flavor formation in onion and garlic is an enzyme action, sulfiting and blanching can not be practiced because of destruction of enzymes. The extreme hygroscopicity of the finished products requires low humidity packaging rooms and moisture-vapor-proof packaging materials. Advantages of dehydrated onion and garlic over their raw counterparts are presented.

Raw Material Considerations

The choice of raw material is always of prime importance in any food product, but this factor has even greater significance when the finished product consists of nothing other than the raw material with water removed which is the case in dehydrated onion and garlic. In our business a poor raw material can not be made acceptable by skillful seasoning or compounding.

Several factors such as availability, quality, and yield determine the variety of raw material chosen. In the dehydration of many fruits and vegetables, market varieties can readily be used since they represent the results of crop research and are in all respects the best available raw source. Unfortunately this is not true in the case of onions. About 85 percent of onion production in the United States is of moderate or low strength varieties. Of the 15 percent of strong pungent onions grown, only about 5 percent are white in color *(5)*. Since a white pungent onion is usually required for drying, the available raw material is limited. As would be expected, there are sound reasons for this situation. Onions are a semi-perishable vegetable and yellow varieties generally hold better in storage. In addition, field yields are considerably higher for the milder varieties. For uses other than dehydration, the market price has no connection with solids content. As a result of these factors, the bulk of onions available in all parts of the country are not suitable for high quality dehydrated products. It is necessary to contract for onions of high solids and pungency at a premium price to compensate the grower for his lower field yields.

Contrary to some current opinion, pungency is not related to latitude, growing temperatures, or length of the growing season. It is interesting to note however, that Platenius and Knott *(4)* found higher strength in onions of several varieties when grown in California as contrasted to Texas, Utah, Massachusetts, Ohio, and New York State. By proper scheduling of plantings, our plant enjoys a season of five months duration without using storage onions.

Crop research has played an important role in developing our present raw material. Common onion varieties vary from 5 percent to 20 percent in solids. As a general rule, low solids onions have larger bulbs as well as larger field yields. Since preparation labor is a big factor in costs, the ideal onion for dehydration is one which has quality of flavor, high solids, and large bulb size. Dr. T. M. Little, our Director of Crop Research, has materially improved our strains in all of the above respects.

[a] Presented before the Ninth Annual I. F. T. Convention, San Francisco, Calif., July 14, 1949.

Some general information about the growing habits of onions may be of interest here. The early growth of the onion plant is almost entirely above ground. Each variety has a specific day length requirement which must be attained before bulb formation starts. If the plant is too small when the day length becomes sufficient, a small onion will result. On the other hand, if the plant is too large at this time, it will probably go to seed—again resulting in an inferior bulb. Thus it is seen that for any geographic location, the temperature and day length requirements will determine which varieties of onions can be grown there successfully. Thus in Louisiana where spring weather is mild, onions with the short day length requirements can be grown successfully, but varieties with longer day length requirements would never form a bulb. In central California, the Louisiana type onions would mature about the size of marbles due to our colder springs. In greenhouse experimentation, it is possible to induce premature bulbing by the use of incandescent lights, or to retard bulbing by covering plants during part of the daylight hours. To my knowledge, neither practice has been done commercially.

Since garlic is sterile, cross breeding and seed selection can not be practiced. Garlic grown in Mexico produces flowers, but no true seed. To date, botanists have not been able to produce garlic by crossing known plants, and its parentage remains a mystery. Lacking true seed, garlic is reproduced by planting whole "cloves" which increase about 20 fold in forming a complete head.

The Enzyme Theory of Flavor Formation in Onion and Garlic

Flavor formation in onion and garlic is an enzyme reaction and the practical application of this knowledge is reflected in their preparation, drying, and use in food products. Cavallito, Bailey, and Buck *(2)* prepared an odorless and tasteless powdered garlic by destroying the enzyme before any cleavage of the precursor could occur. Kohman's work *(3)* traces the short life history of the lachrymating factor. All evidence indicates that the enzyme and precursor cells are separate in the onion bulb or garlic clove. Flavor can be obtained only after the connecting cell walls have been ruptured under favorable pH and temperature conditions. When an onion is sliced, there is a rupturing of cells only at the cut surface. The lachrymator and sulfur-bearing flavoring compounds, in addition to such products as propyl alcohol and propionaldehyde, are formed by the cleavage of the precursor by the enzyme. Unpublished work of Haagen-Smit and his coworkers showed that most of the oils isolated from onion have very low vapor pressure under conditions of dehydration. In fact, shortly after entering the drying tunnels there is almost an absence of onion aroma. In secondary tunnels and drying bins, no aroma is detectable. If our dried slice of onion is powdered after drying to low moisture, the cells which were previously intact are now completely broken and their contents intimately mixed; however, the enzyme reaction can not proceed in the absence of water. The product in this form may be correctly termed "potential" onion flavor. Under proper storage conditions, these factors can lie dormant for years, and the product need only be moistened to start the reaction and form for the first time the onion flavoring oils. This system is in sharp contrast to the essential oils of spices which exist as such in the plant and which can be solvent extracted as well as steam distilled. Similar systems exist in horseradish and also in mustard.

Accepting the enzyme theory of flavor formation, it readily becomes clear why onion and garlic can not be successfully spray dried, blanched, or sulfited. In the first instance, it is necessary to rupture almost all of the cells to reduce the onion to a form which can be sprayed. This wholesale rupturing causes almost complete and instantaneous flavor formation. Subsequent drying results in almost complete loss of low boiling fractions.

Blanching—once almost universally practiced in the dehydration of vegetables—is intended to destroy the very enzymes upon which the flavor formation in onions and garlic depends. A blanched dehydrated onion is of little value as a flavoring material.

Sulfiting can not be practiced for the same reasons, since this chemical inhibits the vital enzyme activity. The treatment of sliced raw onion with the normal sulfite dosage can reduce the resulting flavor of the dried product to one third of the potency of an unsulfited control.

Bacteriology of Dried Onion and Garlic

In the dehydration of vegetables where blanching and sulfiting are practiced, these operations play a significant role in bacterial control. In onion and garlic dehydration, good sanitary practices must shoulder almost the entire burden. The only help is derived from the natural antibiotics which are found in these vegetables. Cavallito and Bailey *(1)* found that an antibiotic, allicin, isolated from garlic, had only one percent of the activity of penicillin; however, it was equally effective against Gram negative organisms which are practically immune to penicillin. Unfortunately, these natural antibiotics are rather selective in their toxicity to common soil, water, and air-borne organisms, but we are fortunate that they are particularly effective against pathogens and *Escherichia coli*. Thorough examination of the dried products in our laboratories has never revealed the presence of this organism.

Packaging

Dried onion and garlic are very hygroscopic and since they are composed chiefly of saccharides, moisture pickup will cause rapid caking of the powdered product, following by decomposition characterized by the familiar "browning reaction." Since moisture control is so vital, we have found it necessary to conduct all milling, inspection, and packaging of our products under low humidity conditions. Needless to say, this would be wasted effort if packaging were not in containers which are practically moisture-vapor proof. At the present time, we use only hermetically sealed tins, triple friction type metal containers and galvanized drums with rubber gaskets and lock rim heads, although other types of containers may be satisfactory.

Advantages of Using Onion and Garlic in the Dried Form

The advantages of using dried onion and garlic take several forms. A few will be discussed briefly.

The market prices of raw onion and garlic are notorious for their tremendous fluctuations from year to year, or even from season to season. The use of dried products produced from contracted raw material smoothes this curve.

Only one crop of onions can be grown yearly, consequently all raw vegetables used between October and April must come from storage where severe bacteriological and physiological breakdowns usually occur. If properly produced, dried forms are stable.

Onions available in any locality throughout a calendar year may vary as much as 300-400 percent in flavor and solids. The manufacturer with rigid quality control must adjust his formulas with almost each change in variety as it becomes available on the market. The dried form can be made from a single uniform variety controlled by furnishing seed to contracted growers.

As mentioned previously, the ability to store well is of more concern to the onion broker than the quality of flavor. He naturally wishes for both, but a decomposed onion can not be sold on the argument that it once had a superior flavor. When onions are processed directly from fields, the emphasis can be shifted to flavor. The Australian Brown is an example of this point. The excellent storage properties are offset by the pronounced bitter flavor which is characteristic of this variety. The generally superior storage stability of yellow onions is largely responsible for the fact that about 85 percent of the total onion production in the United States consists of yellow varieties, all of which contain the pigment quercetin which is itself extremely bitter.

In the case of garlic, sprouting and decomposition take a large percentage of each crop, while metabolic losses will eventually result in the actual disappearance of all except the outer skin and roots.

In processed foods, particle identity can be more easily retained by using the dried form of onion. Some physical change similar to the denaturation of protein takes place in onion tissues upon drying. In many products, identifiable pieces of onion are desirable but processing of the raw vegetables results in "mushing away." When dried onion is substituted, the particles retain their shape and identity adding to the eye appeal, yet only 50 percent of the onion solids which were used in the raw form are required. Once the desired appearance has been established, flavor can be fortified to any extent by the addition of the powdered form. This advantage has proved valuable in such items as Chili Sauce, Corned Beef Hash, and Chili Con Carne.

Use in Processed Foods

Experience—some of it bitter—has taught us that certain usages require special treatment. Here again the enzyme theory of flavor formation plays an important part. Rehydration must be conducted in the optimum pH range for the enzyme. Direct soaking in vinegar or any other acid does not allow complete flavor to form. This is easily corrected by a brief rehydration in water before adding to any acid medium. In most instances, full rehydration is the recommended and logical procedure since the absorption of water replaces the water lost in processing; however, one can see the absurdity of complete rehydration if the product has been or is to be concentrated by the removal of water. In such instances the use of dried onion or garlic can reduce concentration by an amount equivalent to the moisture content of the raw onions or garlic previously used. The percentages of these ingredients are usually quite small, but any shortening of concentrating times is desirable from the quality standpoint. In many instances, processing steps can be eliminated by the use of dried onion or garlic, but each case must be studied individually before recommendations can be made.

An example of a complete change in technique is our present recommended procedure for frying onions. Good fried onions require constant vigilance by the cook or chef to keep from overcooking part and undercooking the balance. We simply recommend lightly sautéing the dry onions in fat and then adding excess water and sufficient salt. From this point on, no attention needs be given until part of the water is absorbed into the onion and the balance driven off as steam at which time the dish is finished. Here we really put the cart before the horse since we fry the dried onions first and then rehydrate. This simple switch has been a boon to harried chefs who already have dozens of dishes to watch simultaneously.

Information about the proper application of dried onion and garlic is constantly increasing and today its uses are much wider and more varied than they were prewar. We feel that any present limitations in their usages will eventually be shown to be the result of our lack of knowledge as to the proper technique of application.

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Palatability of Beef Stored at 0° F. as Affected by Moisture Loss and Oxygen Availability[a, b]

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An important problem in the preservation of packaged frozen meat is loss of palatability during prolonged storage. A study was initiated to determine the relationship of moisture loss and oxygen availability to such palatability losses.

Metal containers were filled to about one-third capacity with ground beef and stored at 0° F. (—18° C.). Atmospheres of low, medium, and high oxygen content under both normal and low relative humidities were maintained during the storage period. Palatability of the samples was judged by a panel after storage of 4 and 7 months.

The data indicated little difference in palatability between lots having low weight losses and those having high weight losses resulting from excessive desiccation. There was a significant decrease in palatability with an increasing oxygen content of the atmosphere surrounding the meat.

Introduction

It is well known that foods do not maintain their original palatability during prolonged storage at freezing temperatures. Recommendations are frequently given as to the maximum time various products may be kept safely in the freezer *(18)*. This loss of flavor is generally a continuous phenomenon and becomes progressively greater with time. Since the major function of any method of food preservation is to increase the storage life of the product, the development of measures which favor the retention of appearance and palatability is of fundamental importance to processors of frozen foods. An understanding of the changes taking place which cause the decrease in desirability to the consumer is necessary to such work.

In the case of meats, reaction between atmospheric oxygen and the unsaturated components of the fat is the most common cause for the development of rancidity *(9)*. There probably are many other changes as well, although the usual storage temperatures largely inhibit attack by microorganisms *(12, 16)*.

Enzymes are not inactivated by the process of freezing *(5, 7, 8)* but are still able to catalyze decomposition of lipids *(1, 3, 13)* proteins *(1, 2, 13)* and carbohydrates *(8, 13)* in the solid state. Lowering the temperature merely reduces the rate of chemical change *(5, 10)*. Furthermore, a variation in the temperature of a biological material may change not only the quantity of degradation, but the course of reaction as well *(11)*. Thus, in the cold storage of potatoes at 0° C., the breakdown of starch to glucose is retarded less than is the respiration of glucose to carbon dioxide and water, causing a buildup of glucose in the tissue *(20)*. In other cases, the accumulation of such intermediates may cause a bad taste in the food. The concentration of oxygen as it determines the oxidation-reduction potential of the medium also affects the activity of some enzymes *(14)*.

A number of preventive measures, such as blanching of vegetables before freezing *(4, 5, 7)*, addition of ascorbic acid to fruits, and use of special packaging materials for meats are in present use. The basic reasons for the greater protective value of the latter are not so well understood, although it has been found that they usually show a low permeability to moisture vapor *(6, 19)*. A number of writers have suggested that the wrapping material used for frozen meats should be impermeable to oxygen as well *(19)*. However, to the author's knowledge, there is no experimental evidence available on which to base such conclusions.

It has previously been found that storage of ground beef *(15)* and adequately processed green beans *(17)* resulted in little loss of flavor during periods in which the decrease in weight exceeded five percent. Therefore a study was initiated to determine the relationship of moisture loss and oxygen availabilty to the decline of palatability during freezer storage.

Experimental Procedure

The meat used for this study was a grade U. S. Good beef round obtained from a local locker plant. It was cut from the carcass and ground twice through the medium sized die of a power machine in the morning of the day of packaging. While holding the bulk of this ground beef in a refrigerator, portions were put through a home grinder using a die having ½ by 5/16 inch elliptical holes. The meat, now in the form of short strings about ⅜ inch in diameter, was accurately weighed on a triple beam kilogram O'Haus balance into 160 gram portions. These were loosely packed in No. 2 tins which were subsequently sealed and treated as described below. It is important to note that the total volume of a No. 2 tin is 600 cc. and the space occupied by the sample was about 150 cc., giving rise to the ratio 3 volumes of air space to one volume of meat.

The experiment was intended to provide six different storage conditions, namely, three levels of oxygen availability, low, normal, and high as indicated in Table 1, with high moisture loss for alternate samples. The low level of oxygen availability was attained by replacing the air in the tin with commercial nitrogen, the normal level by sealing the meat with air, and the high level by leaving the tin open to the atmosphere. An inert desiccant (Desiccite No. 25, Filtrol Corp., Los Angeles, California) was used to attain high moisture losses. Eight identical samples were prepared for each condition in the following manner:

1. The tin was sealed with a lid having in its center a ⅛ inch diameter hole covered with a ¾ inch square

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[b] Paper No. 2493 of the Scientific Journal Series of the Minnesota Agricultural Experiment Station.

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TABLE 1

Moisture Loss and Palatability of Ground Beef Stored at 0° F. (—18° C.) Under Three Levels of Oxygen Availability

Lot No.	Oxygen [4] Availability	Moisture Loss Ave. percent	Palatability Score-total	Palatability percent based on control = 100
1	Low	0.3	255	96.2
2	Low	5.2	221	83.5
			476	90.0
3	Normal	0.4	207	78.2
4	Normal	5.7	208	78.5
			415	78.4
5	High	0.0	175	66.2
6*	High	0.0	171	64.6
			346	65.4
Control	.....	0.0	265	100

[4] Obtained by methods given in the text.
* Note same as Lot 5 in both oxygen and moisture loss.

of plastic locker tape. This lot was then placed in a zero deg. F. home freezer together with the other lots. Five days later, each member of the lot was singly removed from the freezer, its atmosphere replaced with commercial nitrogen gas, and quickly returned to the freezer to avoid a rise in the temperature of the contents. After removing the tape, the opening in the lid was connected to a tee-tube by means of a one-hole rubber stopper used as a gasket. The second leg of the tee was attached to a vacuum pump and the third to a nitrogen tank. The tin was evacuated to a pressure of one mm. Hg., the pump stopped, and nitrogen admitted until atmospheric pressure was again reached. This step was performed a total of three times. As soon as the rubber stopper was removed, the hole was retaped, the tape covered with a layer of Silicone stopcock grease, and a one inch square of sheet metal taped in place over this. Such a seal had previously been tested and found very effective.

2. This lot was treated the same as the first but each sample of meat contained a flat packet of desiccant placed in its center. This packet was prepared by wrapping 30 grams of Desiccite in a 12 cm. circle of No. 1 filter paper and covering with one layer of cheesecloth.

3. Each tin was simply sealed with a solid lid and placed in the freezer.

4. This lot contained a packet of desiccant as described for Lot 2 and was sealed in same manner as Lot 3.

5. The lids used for closing the tins in this lot each had four $\frac{3}{16}$ inch holes punched in them. In addition a piece of filter paper was taped to the underside of each lid in such a way as to allow free passage of gases. This was moistened in order to prevent desiccation of the meat during storage in the freezer.

6. The tins in this lot were also closed with lids having holes as described for Lot 5, but the moistened paper was omitted. It should be pointed out that a desiccant was not used in these samples. It was believed that moisture would be lost by the frozen meat and pass to the outside atmosphere through the openings in the container.

A series of eight controls were obtained by tamping portions of the same ground meat into No. 1 tins and sealing to give a solid pack of 300 grams occupying the total volume of the container. This lot was otherwise handled the same as the others.

The meat was judged for palatability by a taste panel of five persons, two of whom may be considered experienced judges and the remainder as being representative of the goneral public. One control plus one each of the six test lots were judged at a given time. Four sets of samples were tested on different days after about four months storage at 0° F. (—18° C.) and the other four sets after about seven months.

Two hours before the scheduled judging time, the samples were removed from the tins, reweighed, packaged in two layers of 0.0015 aluminum foil, and set out to thaw at room temperature. They were then removed from the foil, broiled until medium done in a gas oven, and recoded. Each judge gave an individual sample a score from zero to ten, the larger values indicating a greater preference.

Results

The average percent moisture loss and the total palatability scores for the samples in the control and each test lot are given in Table 1. The total palatability scores in column 4 show a marked decrease with each increase in oxygen availability. The combined totals for Lots 1 and 2, Lots 3 and 4, and Lots 5 and 6 are 476, 415, and 346, respectively. An inspection of the values for moisture loss, column 2, shows that Lots 2 and 4 lost an average of 5.2 and 5.7 percent weight, respectively. According to the plan of the experiment, it was expected that Lot 6 would show appreciable desiccation, but this lot was found to have no more weight loss than Lots 1, 3, and 5. Consequently, Lot 6 must be considered as identical to Lot 5 and cannot be used for desiccated against non-desiccated comparisons within oxygen levels. However, neither Lot 2 nor Lot 4 shows much difference in total palatability score as compared to their undesiccated counterparts, Lots 1 and 3, respectively. These desiccated samples had the usual dull grey color and typical appearance of severe freezer burn.

These results become more evident when the total score for each test lot is calculated as a percent of the total score for the control series. Thus, in column 5, combining the two lots at each oxygen level gives 90.0, 78.4, and 65.4 percent for the low, normal, and high levels, respectively.

The data were analyzed using the statistical analysis of variance technique, assuming moisture loss in Lot 6. The results show that the variability due to oxygen availability was significant at the one percent level. However, conclusions as to effect of desiccation cannot be based on this analysis because the assumption was invalid. Therefore the data were set up in a two by two table, using only the palatability scores for Lots 1 through 4. An analysis of variance using data for these four lots showed oxygen availabity to be significant at the one percent level, while variability due to moisture loss, judges, samples, and interactions were statistically insignificant.

Discussion

The results indicating that a weight loss of about five percent by the ground beef during storage at 0° F. (—18° C.) had no significant effect on the palatability of the meat are only of academic importance. From the practical standpoint, moisture loss from a frozen product is undesirable due to the adverse effect on appearance and texture as well. It also represents an economic loss to the commercial processor of frozen foods.

The highly significant differences found in the palatability of frozen meat stored in atmospheres containing different concentrations of oxygen serve to emphasize the adverse effect this gas may have on foods at low temperatures. Thus, frozen food packaging materials should be specified as having a very low rate of oxygen, as well as moisture, transmission. In order to allow a more rapid evaluation of various materials for use in frozen food packaging, a method of determining their oxygen permeability under conditions closely simulating those obtaining in actual practice should be made available.

It was noted above that the air in the tins of Lots 1 and 2 was not replaced with nitrogen until several days after the samples were well frozen. Also, all samples were thawed under identical conditions. Thus, any differences due to oxygen between these lots and those packed under a normal atmosphere must have been brought about while the temperature of the meat was at 0° F. (—18° C.). An experiment was carried out to give visual evidence that oxygen can cause changes in meat at this temperature. Lean beef was ground once and formed into patties three inches square and ¼ inch thick. These were immediately wrapped in two layers of 0.0015 aluminum foil, allowed to stand at room temperature for 15 minutes, and then placed in the freezer. After one day the foil was removed from one surface of each of four packages which were then placed in No. 2 tins loosely covered with a lid containing four ⅜ inch holes. At the end of one month the color of the exposed surfaces was compared with that shown by several freshly unwrapped patties. Whereas the latter were purple-red in color, the former were a light red shade. Removal of the foil from the other surface of each test package showed it to have the purple-red color. When the patties were cut in two it was seen that the light red color penetrated to a depth of about 1/16 inch. This color change is probably due to the conversion of hemoglobin to oxyhemoglobin.

Although it is commonly believed that a food left open to the atmosphere during freezer storage will become desiccated, the samples of Lot 6, stored in tins having holes punched in them, showed no weight loss. This was checked by weighing the four patties discussed in the preceding paragraph before and after the months storage in similar tins. They showed an average of one percent moisture loss, far less than one would expect for such a large ratio of exposed surface area to volume. In these experiments, apparently there were other factors involved in this phenomenon.

Summary

Ground beef showed highly significant differences in palatability between lots stored at 0° F. (—18° C.) for periods up to seven months under atmospheres containing low, normal, and high concentrations of oxygen with the scores uniformly decreasing in that order. Moisture losses of five percent caused little decrease in palatability scores.

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Observations on the Oxidase of Asparagus

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Peroxidase and other oxidizing enzymes are convenient and apparently reliable indicators of the keeping quality of frozen vegetables. This paper evaluates the stability of the oxidizing enzymes of asparagus under the influence of various ions and reagents.

It was demonstrated by Joslyn and Bedford *(7)* that there is good correlation between retention of flavor of frozen asparagus in storage and degree of inactivation of peroxidase by blanching before freezing. They found that asparagus showing even a slight positive test for peroxidase developed in several months a noticeable off flavor. In our own experiments conducted in 1939-1941, we confirmed their observation. It had been known since the beginning of the frozen pack vegetable industry, about twenty years ago, that raw unblanched vegetables of most varieties, including asparagus, develop stale or hay-like odors and flavors in freezing storage.

In a survey by one of us (Cruess) in 1946 *(2)*, it was found that samples of frozen asparagus taken from local grocery stores in January and February and showing moderate to strong peroxidase reaction possessed a hay-like flavor and odor. Those that showed a negative test for peroxidase were normal in flavor and odor. All of the samples had been packed commercially eight to ten months previously. Packers must assume that some of each year's pack will be at least a year old before it is consumed; hence blanching must be adequate to prevent serious flavor deterioration for such a period.

It is not known whether peroxidase and other oxidizing enzymes are responsible for the development of off flavors; but they are convenient and apparently reliable indicators of the keeping quality of frozen vegetables.

Joslyn and Bedford *(7)* found, as had others, that catalase of asparagus is destroyed at a much lower temperature than the peroxidase and that asparagus blanched sufficiently to destroy catalase, but not peroxidase, becomes stale and hay-like in flavor in freezing storage.

Joslyn and Bedford (previous citation) found that the aldehyde content of raw and of inadequately blanched asparagus increased in freezing storage and suggested that aldehyde content could be used as another criterion of adequacy of blanching.

In our research we were more interested in the properties of the oxidizing enzymes of asparagus than in frozen asparagus.

Preparation of Enzyme

Solution. For some of our experiments it was desirable to have a solution containing the oxidizing enzymes and catalase. The stalks were washed, coarsely ground in a kitchen food grinder, and were then disintegrated in a Waring blendor with about three times their weight acetone. The mixture was filtered and the residue again blended with acetone; filtered and blended a third time with acetone. After filtration, the solid residue was air dried to remove acetone and was extracted with phosphate buffer at pH 6.0 approximately equal in weight to that of the raw asparagus. The resulting solution was roughly filtered.

Method of testing peroxidase activity. In measuring peroxidase activity quantitatively, measured amounts of dilute guaiacol and H_2O_2 solutions were added to a measured volume of the purified enzyme preparation, appropriately diluted and mixed. After a definite period of time at 30° C., the reaction was stopped by adding 2 cc. of glacial acetic to 10 cc. of the solution. The depth of color of the solution was then read in an Evelyn photocolorimeter at 5200 Ångstroms, the approximate tint of the oxidized guaiacol indicator.

In most of the quantitative tests guaiacol and H_2O_2 were used although benzidine and gum guaiac were used in some cases with H_2O_2 as confirming tests.

Qualitative enzyme tests. Qualitative tests for catalase on pieces of cut raw asparagus with dilute H_2O_2 were strongly positive; for peroxidase with 0.30 percent H_2O_2 and 0.5 percent solutions of guaiacol, catechol, benzidine, gum guaiac, pyrogallol and several other peroxidase indicators were also very positive.

Qualitative tests for oxidase without added H_2O_2 on the cut surface of the asparagus were faintly positive after 15 minutes' exposure with dilute gum guaiac, benzidine and guaiacol solutions and very faint to doubtful with dilute catechol solution. Joslyn and Bedford reported a faint positive reaction of oxidase with gum guaiac solution. The oxidase reactions were very much less pronounced than those usually obtained with apple, peach, apricot, prune and some other fruit tissues. In this connection "oxidase" is taken to mean the enzyme that gives positive reactions with oxidase indicators without added H_2O_2.

Catalase activity with dilute H_2O_2 was quite marked.

In the purified enzyme solution previously mentioned peroxidase activity was very strong, catalase moderately strong and oxidase tests were negative. Evidently the small amount of oxidase in the asparagus tissues was lost during preparation of the enzyme solution.

Joslyn and Bedford *(7)* reported slight and erratic evidence of ascorbase activity in asparagus. We added 100 mgms. per 100 cc. of ascorbic acid to the juice expressed from ground asparagus and also to the purified enzyme solution. Titrations at intervals of 1, 3 and 8 hours with dilute standard iodine solution gave no evidence of decrease in ascorbic acid. Consequently, we concluded that in the asparagus used by us ascorbase was absent or present in very feeble concentration. Fellers et al. (1934) noted very little ascorbase activity in asparagus used in their freezing experiments.

Death temperatures studied. In our recent experiments, catalase in our purified enzyme solution, as

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judged by evolution of O_2 from dilute H_2O_2 solution, survived 60° C. for 5 and 10 minutes although after 10 minutes at 60° C. its activity was low. It also survived 5 and 10 minutes at 65° C. At 68° C. it survived 5 minutes, but not 10 minutes' heating. It was inactivated at 71° C. in 5 minutes as well as in 10 minutes. The pH value of the solution was 6.0. Possibly the death temperature of the catalase would be affected by the pH value of the medium, since Fong and Cruess *(4)* found that the death temperature of peroxidase of fruits was highest in the range of pH 5 to 7; and at lower pH values the death temperature decreased with decrease in pH.

The asparagus peroxidase in our purified enzyme preparation survived 93° C. for 5 and 10 minutes, but not 15 minutes. The peroxidase in raw juice expressed from coarsely ground asparagus survived 5 minutes at 93° C., but not 10 minutes. Possibly the slightly lower pH value of the raw juice, approximately 5.0, compared with that of the purified enzyme solution, pH 6.0, accounted for the difference in resistance.

At temperatures of 90° C. and 87° C. the peroxidase in the purified enzyme solution survived 10 minutes' heating; that in the raw juice survived 5 minutes, but not 10 minutes' heating.

The peroxidase in the purified enzyme preparation survived 5 minutes at 100° C., but not 10 minutes. That in the raw juice was inactivated in 5 minutes at 100° C.

The death temperature of asparagus peroxidase was found to be higher than that of garlic, 90° C. in 5 minutes (Sugihara and Cruess, *8*); olive peroxidase, 80° C. in 5 minutes at pH 6.7; and string bean peroxidase, 90° C. in 2 minutes, Bedford and Joslyn *(1)*. Apricot peroxidase, according to Fong and Cruess *(4)* was inactivated in 5 minutes at 40° C. at pH 2.6; 60° C. at pH 3.2; 80° C. at pH 3.5; 93° C. at pH 4.0; 98-100° C. at pH 7.0 and 70-75° C. at pH 10. Similar results were obtained with the peroxidases of peaches, tomatoes and dates. In other words, at the pH value of our enzyme preparation, their death temperatures did not differ greatly from that of asparagus.

Effect of H_2O_2 concentration. Using the purified enzyme solution with guaiacol as the indicator, the H_2O_2 concentration was varied from .001 to .05N and the peroxidase activity measured as previously described. Table 1 gives the data.

The optimum range appeared to be between .0075 and .001N. If the concentration of H_2O_2 is too high, the enzyme's activity is lowered. Therefore, the H_2O_2 concentration is not a matter of indifference.

Effect of enzyme concentration. The rate of oxidation of the indicator by H_2O_2 varied with the enzyme concentration in a practically straight line relationship. The data are given in Table 2, in which first sample represents the purified enzyme solution diluted so that the enzyme content was 60 percent of the original. Its activity was taken as 100.

TABLE 1

Effect of H_2O_2 concentration on activity of asparagus peroxidase

Normality of H_2O_2	Relative activity
.05	20
.04	23
.03	32
.02	44
.01	67
.0075	74
.005	91
.003	100
.001	80

Effect of substrate concentration. Maximum rate of color formation was obtained at 0.3 to .05 percent guaiacol concentration. Table 3 gives the data on this factor.

Effect of temperature. The activity of the purified peroxidase was observed at various temperatures between 0.8° and 38° C. Apparently 35° C. was somewhat more favorable than 38° C. In the range 0.8-35° C. the enzyme's activity varied more or less with the temperature. See Table 4.

TABLE 2

Effect of enzyme concentration on asparagus peroxidase activity

Relative enzyme concentration	Percent activity
60	100.0
50	94.0
40	77.8
30	65.8
20	45.2
15	29.7
10	19.6
6	11.0
2	3.6
1	0.6

TABLE 3

Effect of guaiacol concentration on asparagus peroxidase activity

Percent guaiacol	Relative activity
0.5	44
0.4	61
0.3	82
0.2	100
0.1	98
0.075	90
0.050	70
0.030	48
0.010	17

TABLE 4

Effect of temperature on asparagus peroxidase activity

Temperature ° C.	Relative activity
0.8	34
4.4	44
7.2	48
12.2	61
15.0	66
20.0	76
28.1	90
32.3	97
35.0	100
38.0	91

Effect of pH value. The activity of the peroxidase with guaiacol and H_2O_2 was determined at pH values ranging from 3.29 to 7.80. The optimum range was at about pH 4.39-4.8. However, as indicated by Hussein and Cruess *(5)*, other substrates would exhibit other pH optima since the pH effect is probably on the substrate rather than on the enzyme.

Effect of trypsin. Incubation of the enzyme solution with trypsin at 37° C. for 20 hours destroyed all peroxidase activity, indicating that a protein or proteins are associated with the peroxidase's activity.

Effect of cyanide. To portions of a dilute solution of the purified enzyme preparation were added small amounts of NaCN, and their peroxidase activity was observed. The data are given in Table 6.

TABLE 5

Effect of pH on asparagus peroxidase activity with guaiacol as substrate

pH value	Relative activity
3.29	57
3.83	94
4.39	97
4.60	100
4.80	90
5.18	81
5.77	66
6.13	42
6.89	19
7.80	4

TABLE 6

Effect of NaCN on asparagus peroxidase activity

Molality NaCN	Relative activity
.000	100
.0001	97
.0003	90
.0005	85
.0007	68
.001	53
.003	12
.005	5
.008	0

The results indicate that the peroxidase preparation was somewhat more resistant to cyanide than is olive peroxidase (Cruess and Sugihara, *3*), which was completely inhibited at .0002M NaCN; grape peroxidase, completely inhibited at .0004M KCN (Hussein and Cruess, *5*); and guava peroxidase, at .000055M (Jimenez, *6*). It was similar in its response to apricot peroxidase (El-Tabey and Cruess, *9*), which required .01M NaCN to completely inhibit it.

Effect of fluoride. To portions of the purified enzyme preparation were added NaF ranging from .001M to .10M. Inhibition was quite marked and more or less in proportion to the NaF concentration. Thus at .01M NaF the peroxidase exhibited 65 percent of normal activity and at .1M, 23 percent; whereas El-Tabey and Cruess *(9)* found that apricot peroxidase was completely inhibited at .10M.

Effect of NaCl. The chloride ion had a pronounced inhibitive effect, but much less than that of the fluoride. Thus at .17M, the peroxidase exhibited 47 percent of normal activity. At higher concentrations the NaCl interfered with normal behavior of the indicator, guaiacol.

Effect of iron salts. At .0002N ferric ion as chloride, peroxidase activity was 160 percent of normal. At .0001N ferrous ion as sulfate, the activity was 97 percent and at .0005N it was 72 percent. Thus, ferric ion appeared to enhance its activity and ferrous to depress it.

Effect of copper salts. At .0005N cupric ions as copper sulfate, the peroxidase activity was 119 percent and at .001N it was 171 percent of normal, confirming the well known catalytic effect of small amounts of dissolved copper on oxidation in biological systems.

Summary

1. Qualitative tests on the surface of cut asparagus indicated the presence of very active catalase and peroxidase. The tests for oxidase without added H_2O_2 were very faintly positive. Tests for ascorbase in a purified enzyme extract were negative.

2. Peroxidase and catalase were freed from the solid tissue and from considerable acetone-soluble material by disintegrating the asparagus in acetone and filtering three successive times, followed by dissolving the enzymes from the solid air-dried residue in phosphate buffer of pH 6.

3. Catalase in the purified enzyme solution was killed at 71° C. in 5 minutes, but not at 68° C. Peroxidase in the same solution survived 5 and 10 minutes, but not 15 minutes at 93° C. It survived 5 minutes at 100° C., but not 10 minutes. It appears to be somewhat more heat resistant than the peroxidase of other fruits and vegetables studied in this laboratory.

4. H_2O_2 concentration affects the activity of the peroxidase, the optimum range in our experiments being .0075 to .001N.

5. Within the limits used in our experiments the amount of substrate oxidized in unit time varied with the enzyme concentration.

6. The optimum substrate concentration, guaiacol, appeared to be 0.1 to 0.3 percent.

7. Activity of the peroxidase was somewhat higher at 35° C. than at 38° C. and decreased with decrease in temperature in the range used by us, 0.8° C. to 38° C., inclusive.

8. With guaiacol, the optimum pH range of the peroxidase was approximately 3.8-4.8.

9. Trypsin inactivated the enzyme.

10. NaCN exerted a very strong inhibitive action, but less than on several other peroxidases studied previously in this laboratory.

11. Fluoride also exerted a much stronger inhibitive action on the peroxidase than did chloride; but asparagus peroxidase was more resistant than apricot peroxidase.

12. Chloride exerted moderate inhibitive action.

13. Traces of ferric ions and copper ions increased the peroxidase activity; traces of ferrous ions decreased it.

14. Like other peroxidases, the asparagus peroxidase catalyzed the oxidation of a rather large number of indicators, most of them phenolic in character.

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Studies on the Darkening of Orange Juice

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Furfural and probably hydroxymethylfurfural, together with other carbonyl compounds, are found in small amounts in Valencia orange juice stored for 4 to 5 months at 37° C., provided oxygen is present. Furfurals were not observed in the absence of oxygen, nor at lower temperatures. Accumulation of furfurals is therefore not normally appreciable, and the juice differs in this respect from orange concentrate and dried apricot.

Joslyn and Marsh *(3)* observed that "the browning of orange juice involves oxidation as a primary step" and suggested that this was followed by non-oxidative reactions leading to accumulation of brown pigments. Various reactions in a second stage were then considered, including those between amino acids and sugars or sugar derivatives, since nitrogen was a constituent of the brown pigments. Additional evidence that oxidation was involved in browning of orange juice was later obtained by Hamburger and Joslyn *(2)*, Loeffler *(4)*, and Moore, et al. *(5)*. Recent evidence *(1)* indicates that hydroxymethylfurfural and furfural are among intermediates responsible for the darkening of dried apricots. Methods developed for their identification *(6, 7)* were therefore applied to orange juice.

Present findings may be summarized as follows: that furfural and hydroxymethylfurfural are to be found in small amounts in juice incubated for four to five months at 37° C., provided oxygen is present; that certain other carbonyl compounds are also found, comparable with those described by F. H. Stadtman *(6)*; that the furfurals do not appear in detectable quantity at lower temperatures (0 to 25°), regardless of browning. On storage for a year or more at lower temperatures, however, it seems possible that furfurals or other aldehydes may form or accumulate in detectable quantity in the presence of preservatives such as monochloracetic acid or sodium benzoate.

Experimental: Valencia oranges from the local market were halved, extracted by burring, and the juice strained through an aluminum screen and then through muslin. The juice was packed in 8 ounce bottles in two series, full and ⅔ full (i.e. with 5 and 85 ml. headspace respectively). The bottles were crown-capped, then heated to 85° for 10 minutes, and stored at 0, 25 and 37° C.

A third set was prepared, to compare the whole juice with the separated chromatophore and aqueous fractions. The chromatophores were separated by centrifugation, washed three times with distilled water to remove water soluble components and re-suspended in an equivalent quantity of dilute citric acid adjusted to pH 3.5, the value for the whole juice. The aqueous portion of the whole juice after centrifugation, was decanted and re-centrifuged until virtually colorless. A dilute citric acid solution was also included as a control.

Measurements of light absorption were made periodically as follows:

Visible Region: The Evelyn photoelectric colorimeter was used with a 440 mμ filter. Clear solutions were obtained by centrifuging to remove chromatophores and suspended material; the supernatant was then diluted with an equal volume of 95 percent ethanol and filtered. As darkening progressed, dark-colored material became insoluble in the partially filled bottles and could not be re-dissolved.

The original juice after the above treatment had an optical density (2-log G) at 440 mμ of 0.051. After five months for the full bottles, this had increased to 0.097, 0.143 and 0.215 at 0°, 25° and 37° C., respectively.

Comparable figures at the same temperatures for the ⅔-filled bottles are 0.081, 0.187 and 0.469. It is clear that oxygen is of primary importance at ordinary and elevated temperatures, but because these samples (particularly at 37°) showed precipitated dark pigment, this technique is not useful for extended rate studies.

The chromatophore fraction bleached on storage. This is due to two causes, discussed in a separate paper, first, an actual loss of pigment by oxidation, second, the formation of less strongly colored cis-carotenoids in such an acid medium.

The aqueous fraction, in the absence of carotenoid chromatophores, darkened somewhat more rapidly than the whole juice, presumably owing to lack of competition for the limited oxygen available.

Ultra-Violet Region: Measurements were made on dilutions usually 1:50, of the various samples after centrifugation, with the Beckman DU Spectrophotometer in the range 400 to 220 mμ at 5 mμ intervals. Curves for five samples are shown in Figure 1, of which the sample represented by curve 4 (storage at 37° for 5 months, ⅔-filled) is the only one with a clearly defined maximum about 285 mμ, and inferentially therefore, possibly containing furfurals. A slight maximum *ca* 280 mμ was also found in a curve for the chromatophores, completely bleached after two months at 37° C. If this is due to additional carbonyl compounds, they may conceivably also play a role in the darkening. The aqueous fraction behaved comparably with the whole juice.

By courtesy of Professor G. L. Marsh, a number of samples of Navel orange juice were examined. These had been preserved more than a year at room temperature with 500 p.p.m. monochloracetic acid, sodium benzoate, and benzoate plus sulfur dioxide. The spectra of the benzoated samples were not interpreted owing to interference caused by the characteristic spectrum of benzoic acid. A sample treated with monochloracetic acid, however, gave a curve similar to curve 4 of Figure 1.

Identification of Intermediates: Darkened Valencia juice samples, stored at 37° for 7 to 8 months were pooled and centrifuged; a total of 2 liters (pigment-free) was made neutral with alkali and continuously extracted

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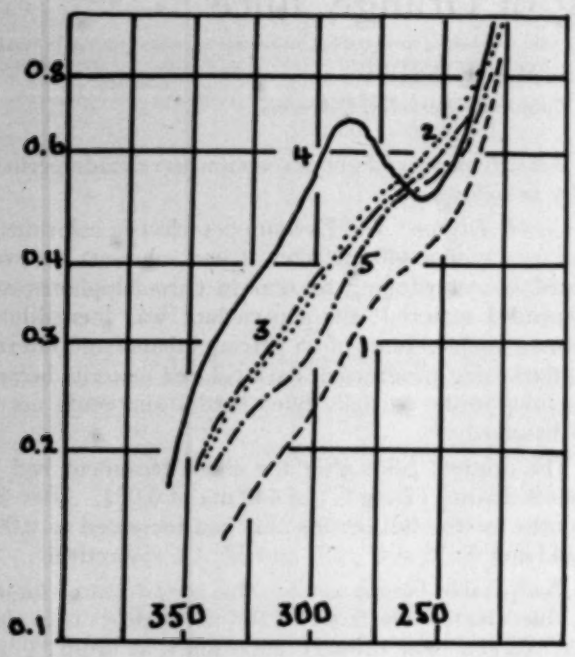

FIG. 1. Ultra-Violet absorption of samples of centrifuged orange juice.

1. Stored full, at 0°, 3 months.
2. Stored full, at 37°, 5 months.
3. Stored ⅔-full, at 25°, 1 year.
4. Stored ⅔-full, at 37°, 5 months.
5. Original, control.

Ordinate, optical densities, at comparable dilutions. Abscissa, wave length in mμ.

for 24 hours with diethyl ether. Compounds responsible for the band at 280-285 mμ were extracted.

Qualitative Tests: a. 2, 4 dinitrophenylhydrazones. A precipitate was obtained with 2, 4 dinitrophenylhydrazine in 2 *N* HCl, indicating the presence of carbonyl compounds.

b. Development of a red color with xylidine and glacial acetic acid indicated *(7)* the presence of furfurals.

c. Chromatography of the dinitrophenylhydrazones: After a pigment zone has been separated by chromatography, its subsequent identification depends upon two steps: comparison of its absorption spectrum with those of known compounds, and a mixed chromatogram. In the case of the dinitrophenylhydrazones, measurements in the region from 220 to 270 mμ create a problem owing to the persistence of benzene in the absorption spectra and the impurities it may contain. Solvent purification is therefore essential. All solvents were purified by distillation prior to use. Benzene was first shaken with *N* NaOH, washed and dried before distillation.

The precipitated hydrazones were dissolved in 20 ml. of 1:1 petroleum ether-benzene, and chromatographed on a column 2.5 cm. diameter, 10 cm. long of 100 mesh Baker's magnesium sulfate. The column was first washed with 100 ml. benzene, then with petroleum ether, and the procedure adopted by Stadtman *(6)* was followed. The main eluate (band I) obtained by developing the 1:1 petroleum ether-benzene solution was evaporated to dryness and re-developed on a second column. Eight zones were obtained, No. 1, with petroleum ether containing 10 percent benzene, No. 2 with 50 percent benzene, No. 3 with benzene, No. 4, with benzene containing 20 percent ethyl acetate, Nos. 5, 6 and 7 with ethyl acetate, No. 8 with absolute alcohol. All eluates were then taken to dryness, re-dissolved in 95 percent ethanol, again dried and re-dissolved. Great care must be taken to remove the benzene which is persistent, as reflected in incorrect absorption spectra. The curves for the eight zones, in 95 percent ethanol, are shown in Figure 2.

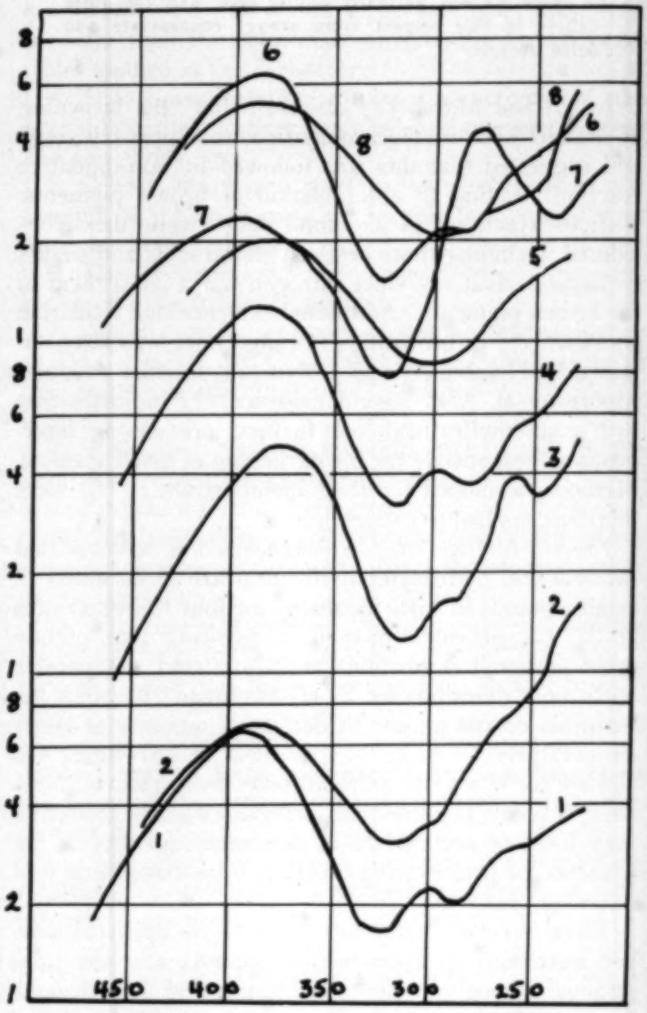

FIG. 2. Spectra of 2, 4 dinitrophenylhydrazones from darkened orange juice.

Numbered in order of increasing strength of adsorption on the column. Curve No. 1 is the furfural derivative, No. 4 the hydroxymethylfurfural derivative.

Ordinate; Optical densities in arbitrary units.

Some of them may be mixtures, but the quantities were too small for further work. Curve 1 resembles the curve for the pure furfural derivative *(6)* and this was confirmed by a mixed chromatogram. Curve 4 is virtually identical with the known curve *(6)* for hydroxymethyfurfural. This was a weak zone on the column and a mixed chromatogram was not possible. Similarities may be noted between curves 2, 3 and 7 of Figure 2 with curves 3, 7 and 9 respectively, reported in apricots *(6)*, but the resemblance must be considered superficial at present.

Discussion

Furfurals have been established among the intermediates in browning reactions of dried fruit extracts. They are evidently formed, in very small amounts, in orange juice stored at elevated temperatures in air. Storage at lower temperatures showed no furfural accumulation, even after 9 months. As a routine measure, the reducing value of the juice was determined by 2, 6 dichlorphenolindophenol titration whenever samples were taken. Joslyn and Marsh *(3)* had found no direct relation between reducing value and darkening (as distinct from a loss in reducing value and onset of darkening) and this may be extended to include a complete lack of correlation between disappearance of ascorbic acid and increase in absorption, *ca* 280 mμ. There is clearly no accumulation of furfural on a scale comparable with that for concentrates and dried apricots. If we assume 20 percent conversion of the ascorbic acid in a sample containing 50 mg. per 100 ml., the curve for sample 4, Figure 1 would be exaggerated 2-fold, taking into account the 1:50 dilution.

Insofar as comparisons between darkening of concentrates and the natural juice are possible, one must concur that the primary step in the darkening of the juice is oxidative. If a comparable step exists in the concentrate, it is obscured, either by virtue of the very nature of the procedure required to prepare the concentrate, or by reactions which proceed only at higher temperatures and more slowly in the more dilute juice. Additional intermediates must be identified before satisfactory mechanisms can be devised in the two cases.

It is worthy of note that no plausible model system has yet been devised which darkens at the rate either of natural juice or of whole concentrate.

Summary

Furfurals may be detected in small amount in orange juice stored in the presence of oxygen at 37° C. Whether formed in quantity or not, they do not accumulate in orange juice on a scale comparable with that found in concentrates.

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The Effect of Borax on the Germicidal Efficiency of Alkali Washing Compounds[a]

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It has previously been shown that the addition of alkaline salts (NaCl, Na_2CO_3, Na_3PO_4, etc.) to a solution of NaOH appreciably increased the germicidal properties of the caustic solution. Sodium metaborate ($NaBO_2$) was found to act in an analogous manner. However, in contrast to the foregoing, the addition of borax ($Na_2B_4O_7$) effects a marked reduction in germicidal efficiency. This is due to interaction of borax with the caustic to form sodium metaborate with a corresponding reduction in concentration of the primary germicidal component, namely, NaOH.

Refillable glass containers as, for example, those used in the milk and beverage industries, must be thoroughly cleaned and submitted to a sterilizing process which will make feasible their re-use. In the beverage and dairy industries glass bottles are cleansed and sanitized with the aid of alkaline washing compounds as detergent and sterilizing agents. Washing compounds generally employed consist of a mixture of sodium hydroxide, which serves primarily as a detergent and germicidal agent, to which are added various amounts of milder alkalies, such as sodium carbonate, trisodium phosphate and in some instances sodium metasilicate, which act in part as water softeners and otherwise facilitate the cleansing processes and particularly rinsing properties of the alkali washing solutions.

Levine and his associates carried on an extensive series of studies in which they have demonstrated that sodium hydroxide is the alkali of primary significance as a germicide, but that, whereas the milder alkalies are of relatively minor significance as germicides, when compared with sodium hydroxide on a normality basis, they do serve to augment appreciably the germicidal efficiency of sodium hydroxide when added to it. They demonstrated further that the addition of salts with a common ion, such as sodium chloride, even though themselves possessing practically no germicidal efficiency in the concentration employed, markedly increased the germicidal efficiency of caustic when added thereto, and that the increased germicidal efficiency was a function of the concentration of sodium ions added in such salts as the chloride, phosphate or carbonate.

Utilization of borax as a substitute for some of the milder alkalies, that is, sodium carbonate or trisodium phosphate, has been repeatedly suggested but evidence

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on either the relative germicidal action of borax or its effect on the sterilizing properties of a sodium hydroxide solution seems not to have been previously investigated.

The objective of this report is primarily to present some data on the effect of the addition of borax (sodium tetraborate $Na_2B_4O_7 \cdot 10H_2O$) and its alkali-reaction product (sodium metaborate, $Na\ BO_2$) on the germicidal efficiency of sodium hydroxide.

I. Technique

In view of the high germicidal efficiency of strong alkalies, such as sodium hydroxide, it was necessary to employ a very resistant test organism. The technique adopted was primarily that previously described by Levine and associates and is described briefly below.

A. *Test Organism.* The test organism employed consisted of spores of *B. metiens,* which was originally isolated from a sample of spoiled ginger ale by Levine, Buchanan, and Lease (1927), a culture of which has been deposited with the American Type Culture Collection.

1. Preparation of Stock Spore Suspension

A suspension of spores of *B. metiens* was prepared by the method described by Rudolph and Levine (1938). Nutrient agar slant cultures which had been incubated for 20 days at 30° C., were washed off in Butterfield's "formula C" water.[4]

The bacterial spore suspension was filtered through sterile filter paper to remove clumps and then heated at 80° C. for 10 minutes to destroy vegetative cells. (Rudolph and Levine demonstrated that such suspensions disclosed no appreciable change in resistance to a commercial calcium hypochlorite solution containing 2,000 p.p.m. available chlorine, during eight months' storage in a refrigerator at 10° C., nor did the resistance differ appreciably from that of dry spores suspended in powdered lactose.)

2. Preparation of Test Spore Suspension

A dilute spore suspension was prepared for each test from this stock spore suspension by adding 10 ml. of this concentrated suspension to 30 ml. of sterile Butterfield "formula C" water which was then stored in the refrigerator until used. This dilute suspension contained about 30 to 40 million spores per milliliter.

B. *Preparation of Test Alkali Solutions.* The test alkali solutions consisted of the following:

1. Sodium hydroxide (N/2) was prepared by dilution of a stock sodium hydroxide solution from which the carbonate had been precipitated by saturation. The solution was standardized by titration to phenolphthalein with a standard hydrochloric acid solution.
2. To N/2 NaOH (2% caustic) was added borax (sodium tetraborate) to a concentration of N/4 (1% calculated as sodium hydroxide alkali equivalent). This solution really constitutes a 3% alkali of which 1% is sodium hydroxide and 2% alkali (calculated as sodium hydroxide) is present in the form of sodium metaborate resulting from the interaction of the borax with the sodium hydroxide.
3. This test solution consisted of 2% sodium hydroxide to which was added 2% sodium metaborate—the product of the interaction of borax (1% calculated as sodium hydroxide alkalinity) with 1% sodium hydroxide.

The objective of employing the above solutions was to determine the effect of the addition of the products of interaction of sodium hydroxide and borax on the germicidal efficiency of a given concentration of sodium hydroxide, in comparison with the effect of adding the same concentration of borax alone to the sodium hydroxide.

C. *Determination of the Germicidal Efficiency of Alkali Solutions.* The test alkali (100 ml.) was placed in a three necked round bottom Woulff flask. The flask was then placed in a DeKhotinsky water bath which had been adjusted to the desired temperature. A motor driven glass stirrer which passed through the middle neck of the flask, constantly agitated the test solution. One neck of the Woulff flask was used to withdraw samples and the other held the thermometer. The alkali solution was maintained at 50° C. ± 0.2° C.

When the test solution had attained and remained at the desired temperature for 5 minutes, 1.0 ml. of the dilute spore suspension (30-40 million spores per ml.) was introduced into the flask so that the concentration of spores in the alkali disinfecting mixture was between 300-400,000 per ml.

At desired time intervals, 5 ml. portions were removed and introduced into 125 ml. Erlenmeyer flasks containing 45 ml. of sterile distilled water, thus immediately stopping the action of both alkali and temperature. Further dilutions were then made in 9 ml. water blanks and the survivors determined by plating on standard nutrient agar, which was incubated at 30° C. and counts made after 18 to 24 hours' incubation.

Determination of the initial count was made in the same manner except that sterile distilled water was employed in place of the test alkali solution. This also served to demonstrate the fact that the temperature, in the absence of alkali, had no germicidal effect.

The time required to effect a reduction of 99.9% in the number of viable spores, called the "Killing Time," was taken as a basis for comparing the germicidal efficiency of the solutions under examination. This "Killing Time" was determined from a plot of the logarithms of survivors against the period of exposure expressed in minutes.

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[4] Butterfield Formula C Water

A. *Stock Solutions*

1. Stock phosphate buffer solution—dissolve 34 gms. KH_2PO_4 in 500 ml. distilled water adjusted to pH 7.2 with 1 N NaOH and made up to one liter.
2. Stock $CaCl_2$ solution—0.10 M (18.3 gms. $CaCl_2 \cdot 4H_2O$ per liter)
3. Stock $MgSO_4$ solution—0.04 M (9.9 gms. $MgSO_4 \cdot 7H_2O$ per liter)
4. Stock $FeCl_3$ solution—0.001 M (0.27 gms. $FeCl_3 \cdot 6H_2O$ per liter)

B. *Formula C Water*

This is prepared from the above stock solutions in the following manner:

To 500 cc. of distilled water add the following quantities of the above stock solutions.

1. Stock phosphate buffer ..... 1.25 ml.
2. Stock 0.1 M calcium chloride solution ..... 2.5 ml.
3. Stock 0.04 $MgSO_4$ solution ..... 2.5 ml.
4. Stock 0.001 M $FeCl_3$ solution ..... 0.5 ml.

Make up to one liter with distilled water.

II. Results

The experimental data are shown in Tables 1, 2, and 3 and the results, as respects times required to kill 99.9% exposed spores, are summarized in Table 4.

The killing times, employing sodium hydroxide (Table 1) ranged from 39 to 43.5 minutes with an average of 41.5 minutes. This value is in excellent agreement with a killing time of 42.5 minutes which was obtained by Levine, Buchanan, Lease, and others.

From the data in Table 2 (summarized in Table 4) it may be seen that the addition of N/4 borax (sodium tetraborate) ($Na_2B_4O_7$) to N/2 NaOH resulted in a marked decrease in the germicidal efficiency of the mixture over the N/2 NaOH alone, as is indicated by increased killing times to 68 to 76 minutes (or an average of 71 minutes). Thus the addition of N/4 borax (1.0% calculated at sodium hydroxide alkalinity) to N/2 NaOH almost doubled the time required for killing 99.9% of the exposed spores of *B. metiens*.

In contrast to the foregoing, it will be noted (Tables 3 and 4) that the addition of N/2 sodium metaborate ($NaBO_2$) (2% alkali calculated as NaOH), which is the product resulting from the interaction of N/4 $Na_2B_4O_7$ (1.0% alkali calculated as NaOH) with NaOH, effected a marked increase in the germicidal efficiency, when added to N/2 NaOH, as is indicated by a reduction of the killing time to 28.5 minutes.

Explanation of the decrease in germicidal efficiency of sodium hydroxide on addition of borax ($Na_2B_4O_7$) whereas the germicidal efficiency is increased on addi-

TABLE 1

Germicidal Efficiency of N/2 NaOH (2% caustic) Against B. metiens Spores at 50° C.

Exposure (Time in minutes)	Experiment A		Experiment B		Experiment C		Experiment D	
	Surviving Bacteria*		Surviving Bacteria*		Surviving Bacteria*		Surviving Bacteria*	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
0	1,375,000	100.0	1,400,000	100.0	1,875,000	100.0	1,375,000	100.0
10	1,110,000	80.0	900,000	65.5	1,500,000	80.0	800,000	58.1
15	650,000	47.3	600,000	43.7	.....	.....	.....	.....
20	325,000	23.6	185,000	13.5	500,000	26.6	115,000	8.4
25	85,000	6.2	60,000	4.4	.....	.....	.....	.....
30	30,000	2.2	45,000	3.3	30,000	1.6	17,500	1.3
35	12,000	0.9	11,000	0.8	.....	.....	.....	.....
40	2,500	0.2	1,750	0.1	9,000	0.5	1,000	0.07
45	.....	.....	.....	.....	1,100	0.06	.....	.....
Killing time (in minutes)	42.		41.		43.5		39.	

* In 5 ml. disinfecting solution.

TABLE 2

*Gemicidal Efficiency of Mixture of N/2 NaOH (2% caustic) and N/4 Borax (calculated as NaOH alkali equivalent) Against B. metiens Spores at 50° C.**

Exposure (Time in minutes)	Experiment A		Experiment B		Experiment C		Experiment D	
	Surviving Bacteria**		Surviving Bacteria**		Surviving Bacteria**		Surviving Bacteria**	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
0	1,650,000	100.0	1,500,000	100.0	1,625,000	100.0	1,750,000	100.0
10	1,625,000	98.5	1,500,000	100.0	1,750,000	107.8	1,500,000	85.8
20	1,100,000	66.6	800,000	53.3	950,000	58.5	1,000,000	57.1
30	550,000	33.3	350,000	23.4	500,000	30.8	450,000	25.7
40	170,000	10.3	100,000	6.7	150,000	9.2	145,000	8.3
50	70,000	4.2	95,000	6.3	30,000	1.9	30,000	1.7
60	16,000	1.0	16,000	1.1	17,500	1.1	17,500	1.0
70	6,000	0.4	1,750	0.12	1,250	0.08	800	0.05
Killing time (in minutes)	76.5		73.0		68.0		68.5	

*After admixture of Borax (sodium tetraborate) with NaOH, the disinfecting solution actually becomes a 3% alkali consisting of N/4 NaOH (1% caustic) and N/2 sodium metaborate (2% alkali calculated as NaOH).

**In 5 ml. disinfecting solution.

TABLE 3

*Germicidal Efficiency of Mixture of N/2 NaOH (2% caustic) with N/2 Sodium metaborate (calculated as NaOH alkali equivalent) Against B. metiens Spores at 50° C.**

Exposure (Time in minutes)	Experiment A		Experiment B		Experiment C	
	Surviving Bacteria**		Surviving Bacteria**		Surviving Bacteria**	
	Number	Percent	Number	Percent	Number	Percent
0	1,750,000	100.0	1,625,000	100.0	1,500,000	100.0
5	1,500,000	85.8	1,125,000	69.3	.....	.....
10	700,000	40.0	500,000	30.8	800,000	53.4
15	165,000	9.4	150,000	9.2	250,000	16.7
20	40,000	2.3	22,000	1.4	65,000	4.3
25	12,500	0.71	9,500	0.6	11,000	0.7
30	650	0.04	800	0.05	1,100	0.07
Killing time (in minutes)	28.5		28.0		29.0	

*After admixture of sodium metaborate with NaOH the disinfecting solution actually is a 4% alkali consisting of N/2 NaOH (2% caustic) and N/2 sodium metaborate (2% alkali calculated as NaOH).

**In 5 ml. disinfecting solution.